JOURNAL OF BACTERIOLOGY, Oct. 2001, p. 5762–5767 Vol. 183, No. 19
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.19.5762–5767.2001

Residue Aspartate-147 from the Third Transmembrane Region of Na^+/H^+ Antiporter NhaB of *Vibrio alginolyticus* Plays a Role in Its Activity

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Received 30 April 2001/Accepted 10 July 2001

NhaB is a bacterial Na^+/H^+ antiporter with unique topology. The pH dependence of NhaB from *Vibrio alginolyticus* differs from that of the *Escherichia coli* NhaB homolog. Replacement of Asp-147 with Glu made high H^+ concentrations a requirement for the NhaB activity. Replacement of Asp-147 with neutral amino acids inactivated NhaB.

All living cells, with only one exception (18), appear to contain Na^+/H^+ antiporters (13). *Escherichia coli* has NhaA and NhaB Na^+/H^+ antiporters (4, 14). These proteins have been purified and reconstituted, and their Na^+ transport activities have been analyzed (15, 20) by using the imposed ammonium gradient method (7). *E. coli* contains the third Na^+/H^+ antiporter gene, *chaA* (3). The triple deletion mutant *E. coli* strain TO114 (Δ*nhaA* Δ*nhaB* Δ*chaA*) is sensitive to Na^+ stress (12). *Vibrio alginolyticus* contains two Na^+/H^+ antiporter genes, *Va-nhaA* (8) and *Va-nhaB* (6). Deduced amino acid sequences of Va-NhaA and Va-NhaB are 58 and 67% identical with NhaA and NhaB from *E. coli*, respectively. There exists no homology between the NhaA and NhaB families. However, either the *Va-nhaA* or *Va-nhaB* gene can restore growth of *E. coli* strain TO114 at high NaCl concentrations. NhaA contains 12 transmembrane regions (TMs) (17, 23). Three conserved aspartic acid residues have been identified in the middle of TM4 and TM5 in both NhaA and Va-NhaA. Their importance for antiport activity has been reported (9). An acidic residue(s) in a TM(s) of Va-NhaB may have a similar function. Va-NhaB has been reported to have a unique topology that contains at least nine TMs with the N terminus inside and C terminus outside, as well as a loop-like region that may fold back into the membrane region (2). Va-NhaB contains 40 negatively charged residues. However, only three amino acids, Glu-74, Asp-147, and Asp-407, are predicted to be located inside membrane regions. Glu-74 is located in the loop-like region and Asp-407 is located in TM8 near the cytoplasmic surface. Only one acidic residue, Asp-147, is predicted to be present in the middle of a TM (TM3). This residue is conserved in all known members of the NhaB family: *E. coli* (M83655), *V. alginolyticus* (D83728), *Vibrio parahaemolyticus* (D83708), *Vibrio cholerae* (AE004265), *Haemophilus influenzae* (U32726), *Pasteurella multocida* (AE006038), *Pseudomonas aeruginosa* (AB037930), *Thermotoga maritima* (AE001757), *Pyrococcus abyssi* (AJ248288), and *Mycobacterium tuberculosis* (Z96072). We have investigated the role of residue Asp-147 in antiport activity. Here we show that the Asp-147 residue plays a role for cation transport of the NhaB Na^+/H^+ antiporter.

Bacterial strains, plasmids, media, and experimental techniques. Plasmid pBD519 contains a *Va-nhaB-phoA* fusion gene in the vector plasmid pPAB404 (1) (Table 1). This fusion gene encodes the truncated Va-NhaB, lacking the nine C-terminal amino acids and fused to alkaline phosphatase (PhoA), resulting in a 959-residue NhaB-PhoA fusion protein (2). Plasmid pGEX-*nhaB* contains a *GST-Va-nhaB* fusion gene in the vector plasmid pGEX-2TK (Pharmacia). This fusion gene encodes glutathione *S*-transferase (GST) fused with truncated Va-NhaB and lacking the three N-terminal amino acids, resulting in the 759-residue GST-Va-NhaB fusion protein. For the construction of pGEX-*nhaB*, the 2-kb *Eco*RV-*Sma*I fragment containing the *Va-nhaB* gene of the pTN1 (6) was cloned into the *Sma*I site of pGEX-2TK in the correct direction. For site-directed mutagenesis, a silent mutation giving an *Afl*II site was introduced by using a two-step PCR procedure into the region upstream of the Asp-147 codon, giving pBD147D (22). Plasmids pBD147X, in which X is G (Gly), T (Thr), M (Met), or E (Glu) and indicates the amino acid residue that replaced the Asp-147 residue, were constructed between the *Afl*II and *Nco*I sites by PCR. The nucleotide sequences from all PCR-generated fragments were verified by sequencing. Plasmid pNB2 containing the *nhaB* gene from *E. coli* was constructed by ligation of the *Eco*RV-*Bam*HI fragment of Kohara phage clone 11G8 with pACYC184 cut with *Eco*RV and *Bam*HI (12). Plasmid pHGB2 containing *nhaB* from *E. coli* was constructed by ligation of the *Xmn*I-*Bam*HI fragment of pNB2 to pHG165 (19), which was cut with *Sma*I and *Bam*HI.

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). Plasmid-containing cells of *E. coli* strain TO114 (Δ*nhaA* Δ*nhaB* Δ*chaA*) (12) were precultured aerobically in a Monod tube at 37°C in medium PYK100Amp, which contained 0.5% polypeptone and 0.5% yeast extract brought to pH 7.0 with KOH, 100 mM KCl, and 50 μg of ampicillin/ml.

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TABLE 1. Plasmids used in this study

Plasmid	Characteristic	Source	Reference
pHG165	*amp*^{r}		20
pTN1	*Va-nhaB* on pHG165	Lab collection	8
pPAB404	*amp*^{r}	W. Epstein	2
pBD519	*Va-nhaB-phoA* on pPAB404	Lab collection	3
pGEX-2TK	*amp*^{r}	Pharmacia	
pGEX-*nhaB*	*gst-Va-nhaB* on pGEX-2TK		This work
pBD147D	Contains silent *Afl*II site	pGEX-*nhaB*	This work
pBD147G	Contains Gly (GGG) mutation at Asp-147 (GAT)	pBD147D	This work
pBD147T	Contains Thr (ACG) mutation at Asp-147	pBD147D	This work
pBD147M	Contains Met (ATG) mutation at Asp-147	pBD147D	This work
pBD147E	Contains Glu (GAG) mutation at Asp-147	pBD147D	This work
pNB2	*E. coli nhaB* gene	H. Kobayashi	14
pHGB2	*E. coli nhaB* gene on pHG165	pNB2	This work

After the OD_{600} had reached a value between 0.4 and 0.6, calculated amounts of cells were collected by centrifugation and then resuspended to an OD_{600} of between 0.04 and 0.06 in PYK100NaXAmp medium, which is PYK100Amp medium supplemented with X representing the millimolar concentration of NaCl. Plasmid-containing TO114 cells were cultured in PYK100Amp medium up to an OD_{600} of 0.5 to 0.7. Cells were harvested by centrifugation at 4°C and washed once with TCDS (10 mM Tris-HCl [pH 7], 0.14 M choline chloride, 0.5 mM dithiothreitol, 0.25 M sucrose) supplemented with 0.5 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride]. Membrane vesicles were prepared by French press lysis of cells in TCDS with AEBSF as described previously (4, 16). Membrane proteins were solubilized in 0.2 N NaOH. Subsequently, protein concentrations were determined by the method of Lowry et al. (5) with bovine serum albumin as a standard. The immunoblot analysis procedure was essentially the same as described previously (2). Membrane vesicles corresponding to 20 μg of protein were solubilized by treatment with sodium dodecyl sulfate (SDS) loading buffer at room temperature for 20 min (11). Solubilized proteins were separated in a 9% SDS–polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). Goat anti-GST antibody (Pharmacia) and anti-goat immunoglobulin G-alkaline phosphatase (PhoA)-labeled antibody (Toyobo) were used for detection of the GST-Va-NhaB and variant proteins.

Na^+-H^+ exchange activity was estimated from quenching recovery of acridine orange fluorescence (7, 16). Reaction buffer consisted of 10 mM bis-Tris-propane (pH 6.5 to 8.5 by HCl) and contained 0.14 M choline chloride and 5 mM $MgCl_2$. Acridine orange (1 μM) and vesicles (40 μg of protein/ml) were added, and the fluorescence intensity of acridine orange was monitored by excitation at 430 nm and emission at 570 nm. For each experiment, 10 mM Tris-D,L-lactate, 10 mM NaCl, and 25 mM NH_4Cl were added in that order. Na^+-Na^+ exchange and net Na^+ efflux activity was measured using K^+-depleted and Na^+-loaded cells prepared as described previously (10). Internal Na^+ and K^+ concentrations at this step were determined by flame photometry as described elsewhere (10). One hundred microliters of Na^+-loaded cells suspended in 0.15 M NaCl with 50 mM HEPES (pH 7.5 by NaOH) were equilibrated with 74 kBq of carrier-free $^{22}Na^+$ for 2 h on ice. For the measurement of $^{22}Na^+$ efflux activity, the $^{22}Na^+$-equilibrated cells (2 μl) were added to the $^{22}Na^+$-free buffer (100 μl), which contained 0.2% glucose and 20 mM KCl. At intervals, the cells were collected by filtration and washed three times with 1.5 ml of chilled 0.15 M choline chloride containing 10 mM Tris-HCl (pH 7.5). Net Na^+ efflux was determined under the same conditions described above without $^{22}Na^+$. Intracellular Na^+ was determined by flame photometry as described before.

Va-NhaB and fusion proteins. TO114 cells containing vector plasmids pGEX-2TK or pPAB404 grew in media containing <50 mM Na^+, but they did not grow in media containing >100

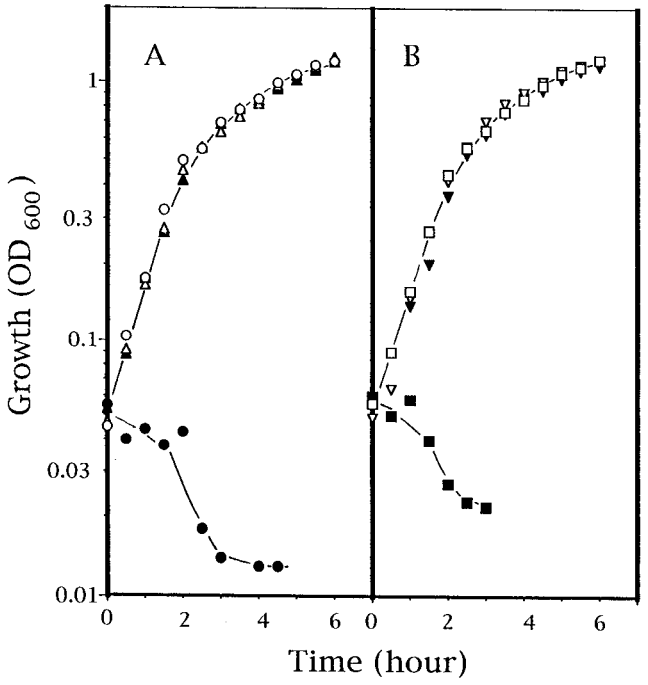

FIG. 1. Growth at high NaCl concentrations of TO114 cells carrying a plasmid with *Va-nhaB* fusion genes. (A) Plasmid pGEX-*nhaB*, containing the *GST-Va-nhaB* fusion gene (△, ▲), and vector plasmid pGEX-2TK (○, ●). (B) Plasmid pBD519 (▽, ▼), containing the *nhaB-phoA* fusion gene, and vector plasmid pPAB404 (□, ■). Cells were precultured in PYK100Amp medium and then cultured in PYK100Amp (open symbols) or PYK100Na200Amp (closed symbols) as described in the text.

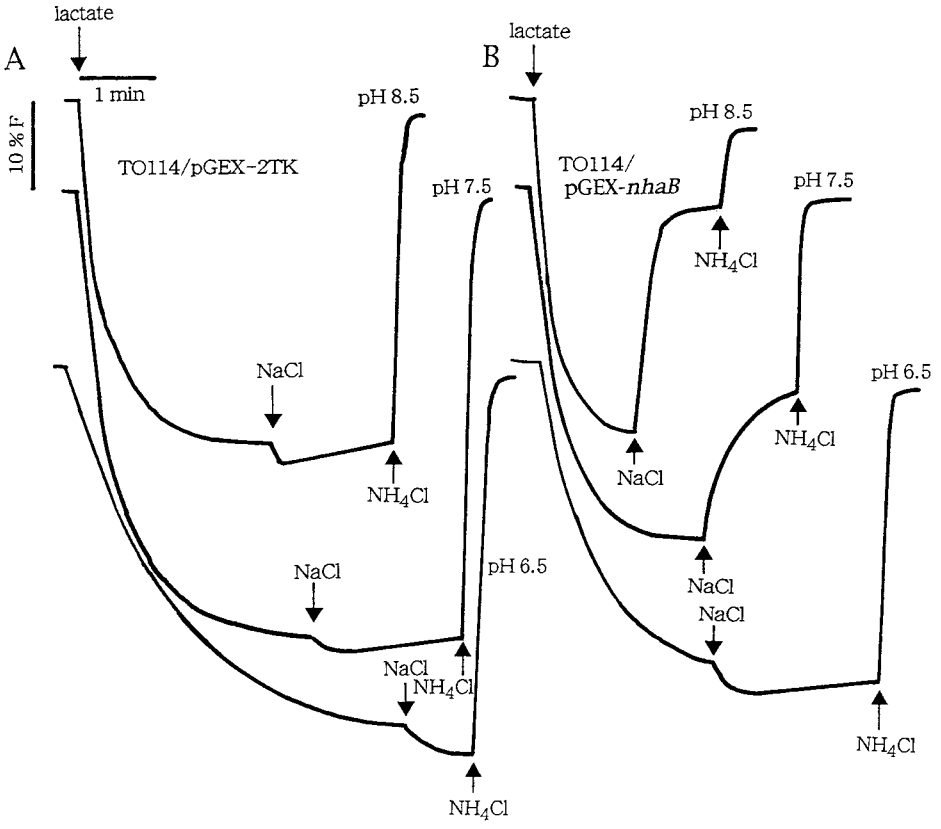

FIG. 2. Detection of Na^+-H^+ exchange activity with a fluorescence quenching assay. French press (everted) membrane vesicles were prepared from TO114 cells carrying vector plasmid pGEX-2TK (A) or pGEX-*nhaB* (B). The ΔpH formation with addition of 10 mM Tris-D,L-lactate (first arrow) was monitored by measuring the decrease of acridine orange (1 μM) fluorescence intensity in medium containing 140 mM choline chloride, 10 mM bis-Tris-propane (titrated with HCl to the indicated pH), 5 mM $MgCl_2$, and membrane vesicles (40 μg of protein). Na^+-H^+ exchange activity was detected by an increase in fluorescence intensity after addition of 10 mM NaCl (second arrow). NH_4Cl (25 mM) was added in order to abolish ΔpH completely (third arrow).

mM Na^+, such as PYK100Na200Amp (Fig. 1). By contrast, TO114 cells harboring plasmid pGEX-*nhaB* or plasmid pBD519, which contain the fusion genes *GST-Va-nhaB* and *Va-nhaB-phoA*, respectively, grew in the PYK100Na200Amp medium (Fig. 1). We did not detect any difference in growth behavior between TO114(pGEX-*nhaB*), TO114(pBD519), and TO114(pTN1). pGEX-*nhaB* and pBD519 contained *Va-nhaB* fusion genes, and pTN1 contained the complete *Va-nhaB* gene (data not shown). Apparently, neither the GST or PhoA fusion nor the absence of the N-terminal 3 amino acids or C-terminal 9 amino acid residues influenced the Na^+/H^+ antiport activity of Va-NhaB.

This conclusion was supported by results from fluorescence quenching experiments using inside-out (everted) membrane vesicles. Addition of lactate energizes the vesicles, leading to intravesicular accumulation of H^+ due to the activity of the electron transport system. Intensity of acridine orange fluorescence decreases upon vesicular acidification due to dye accumulation inside the vesicles. Figure 2 shows the usefulness of this method. In vesicles derived from TO114 cells lacking Na^+/H^+ antiporters [TO114(pGEX-2TK)], the dye fluorescence was quenched after the addition of lactate, and then there was no enhancement of fluorescence after addition of Na^+ at pH 6.5, 7.5, or 8.5. Addition of NH_4Cl caused complete dequenching of fluorescence because NH_3 passes through the membrane in its neutral form and becomes NH_4^+ by binding a proton inside the vesicles, thereby abolishing the H^+ concentration gradient across the vesicle membrane. In contrast, membrane vesicles from strain TO114(pGEX-*nhaB*) exhibited an increase in the fluorescence due to addition of Na^+ at pH 7.5 or 8.5 (Fig. 2), and these responses were the same as those in vesicles derived from TO114(pTN1) (data not shown). These results indicate Na^+-H^+ exchange activity of vesicles containing the GST-Va-NhaB fusion protein. At pH 6.5, Na^+-H^+ exchange activities of both Va-NhaB and GST-Va-NhaB in everted membrane vesicles were undetectable (data not shown and Fig. 2, respectively). However, Padan and Schuldiner reported that NhaB from *E. coli* is active at pH 6.5 (13). In order to resolve this discrepancy, we cloned *nhaB* from *E. coli* into plasmid pHG165, giving plasmid pHGB2. Inside-out membrane vesicles prepared from strain TO114(pHGB2) did show Na^+-H^+ exchange activity at pH 6.5 to 8.5 (data not shown), confirming the results of Padan and Schuldiner (13). We conclude that Ec-NhaB and Va-NhaB have different profiles of activity in response to intracellular pH. At present, the structural basis for this difference is not known.

Variants of Asp-147. Asp-147 is the only acidic residue predicted to be located in the middle of a transmembrane stretch

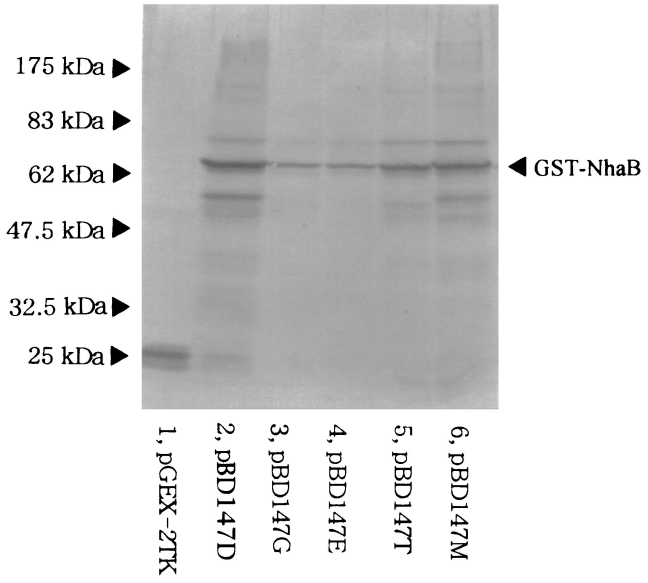

FIG. 3. Detection of GST-NhaB and GST-NhaBD147X variant proteins in the membrane fraction. The membrane fraction from TO114 cells carrying pGEX-2TK (lane 1), pBD147D (lane 2), pBD147G (lane 3), pBD147E (lane 4), pBD147T (lane 5), or pBD147 M (lane 6) were prepared as described in the text. Proteins (20 μg) from this fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting. GST-NhaB and Asp-147 variant proteins were detected immunologically using anti-GST antibody as described in the text.

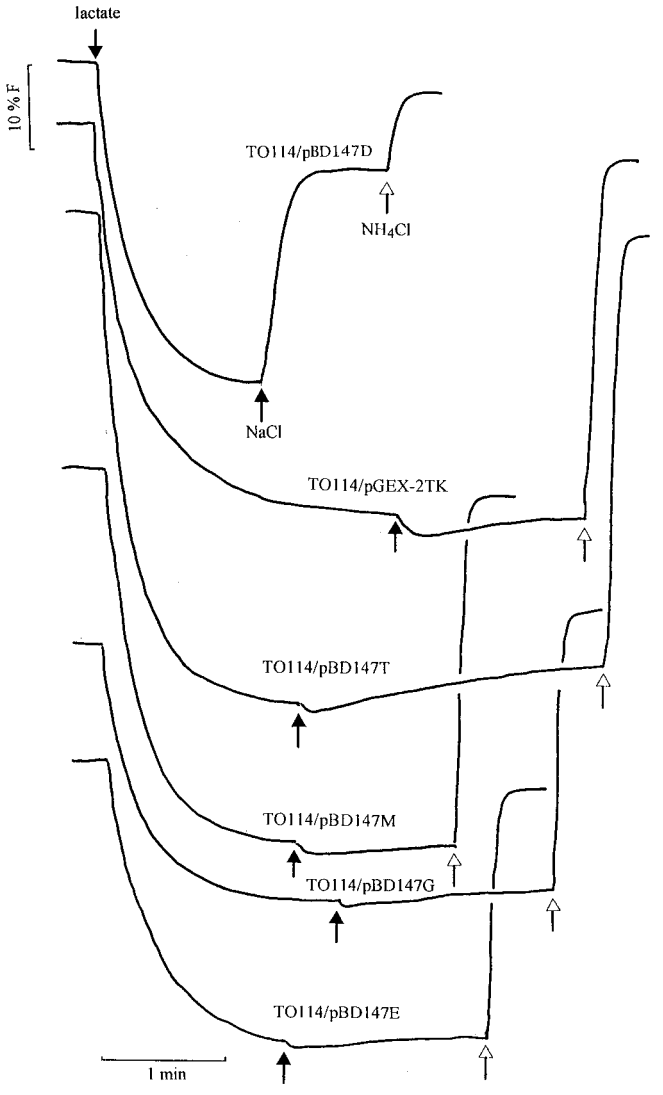

FIG. 4. Detection of Na^+-H^+ exchange activities of vesicles expressing GST-Va-NhaB or its residue-147 variants. Membrane vesicles were prepared from TO114 cells carrying pBD147D (line 1), pGEX-2TK (line 2), pBD147T (line 3), pBD147 M (line 4), pBD147G (line 5), or pBD147E (line 6). The experiment was carried out at pH 8.5 as described in the legend to Fig. 2. Fluorescence quenching was initiated by the addition of 10 mM lactate. The upward arrow with a filled head shows the moment at which 10 mM NaCl was added; the open head indicates the addition of 25 mM NH_4Cl.

of Va-NhaB (2). The importance of Asp-147 for the function of Va-NhaB was investigated using GST-tagged Va-NhaB. Asp-147 was replaced with Gly, Thr, Met, and Glu. French press membrane vesicles were prepared from TO114 cells containing pBD147X or the vector plasmid pGEX-2TK. Membrane vesicles were subsequently assayed for the presence of the fusion protein by immunodetection. All GST-Va-NhaB proteins were present in the membrane fraction (Fig. 3). They showed a migration distance on the gel corresponding to a molecular mass of about 65 kDa, which is smaller than the predicted mass of 84 kDa. This difference is probably due to the fact that Va-NhaB is an integral membrane protein. Fluorescence quenching experiments were done using these vesicles. All variants showed no significant Na^+-H^+ exchange activity both at pH 8.5 (Fig. 4) and at pH 7.5 (data not shown). These results show the importance of Asp-147 for Na^+-H^+ exchange activity. TO114 cells containing GST-Va-NhaB or its Asp-147 variants grew with identical rates in PYK100Amp medium (data not shown). On the basis of fluorescent experiments, one would predict that all Asp-147 variants would have lost their activity to complement TO114 for NaCl tolerance. This prediction was true for the Gly, Thr, and Met variants. Surprisingly, the variant with the acidic residue Glu complemented TO114 growth in PYK100Na200Amp at pH 7.0 (Fig. 5A). TO114 cells with pBD147D or pBD147E grew with essentially the same rate in PY medium up to 500 mM Na^+ (pH 7.0) (data not shown). To understand the discrepancy between the results shown in Fig. 4 and 5A for the Glu-147 variant, the effect of external pH on growth was tested (Fig. 5B). TO114(pBD147D) grew well in medium containing 100 mM Na^+ at pH 8.5. However, cells carrying pBD147E did not grow under these conditions. Apparently, in intact cells the Glu-147 variant is active at neutral external pH but inactive at pH 8.5. Possibly, Asp-147 is involved in the H^+ binding site for Na^+-H^+ exchange activity and the Asp and Glu residues have different pK_a values for binding of external H^+ in the NhaB molecule.

$^{22}Na^+$ loading of plasmid-containing cells of strain TO114 was carried out. In the absence of all three Na^+/H^+ antiporters, it is not known by which mechanism this strain accumulates Na^+ ions. On the other hand, recent experiments indicate that strain TO114 contains a rapid Na^+ uptake system (N.

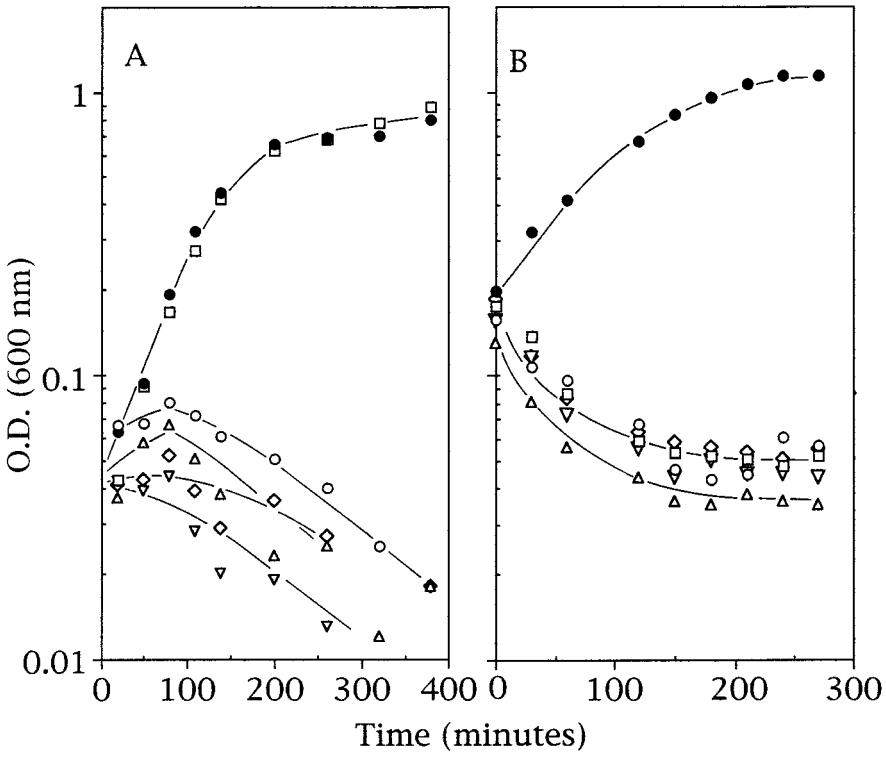

FIG. 5. Effect of an amino acid alteration of the NhaB residue Asp-147 on growth of TO114 cells at high NaCl concentrations. TO114 cells carrying pGEX-2TK (○), pBD147D (●), pBD147E (□), pBD147G (△), pBD147T (◇), or pBD147 M (▽) were precultured in PYK100Amp medium and then cultured in PYNa200Amp (pH 7.0) (A) or PYNa100Amp (pH 8.5) (B).

Tholema, and E. P. Bakker, personal communication), and it may well be that this system is involved in the Na^+-loading process. Internal cation concentrations of the loaded cells were about 900 to 1,000 nmol of Na^+/mg of protein and less than 30 nmol of K^+/mg of protein for TO114 cells with pGEX-2TK or pBD147D. $^{22}Na^+$-Na^+ exchange was measured by diluting $^{22}Na^+$-loaded cells 50-fold into Na^+-containing buffer from which $^{22}Na^+$ was absent and then determining the $^{22}Na^+$ content of the cells as a function of time (Fig. 6). TO114(pGEX-2TK) cells lost their $^{22}Na^+$ relatively slowly: a 50% reduction of their $^{22}Na^+$ content occurred in about 6 min (Fig. 6). Under these conditions, Na^+ and K^+ levels in the cells remained constant according to flame photometry results (data not shown), indicating that the $^{22}Na^+$ efflux, as illustrated in Fig. 6, represented $^{22}Na^+$-Na^+ exchange, possibly via the Na^+ uptake system described above. $^{22}Na^+$-loaded cells of strain TO114 containing the Va-NhaB-encoding plasmid pTN1, pGEX-*nhaB*, or pBD519 all lost 50% of their $^{22}Na^+$ within less than 1 min (Fig. 6). Under these conditions, the cells extruded net Na^+ and took up net K^+ with rates of about 50 to 100 nmol · min^{-1} · mg of $protein^{-1}$ (data not shown). Since these rates are about 1/10 of the $^{22}Na^+$-Na^+ exchange rate by these cells (Fig. 6), we conclude that the extremely rapid $^{22}Na^+$ efflux shown in Fig. 6 for these cells represents $^{22}Na^+$-Na^+ exchange via Va-NhaB. The GST-Va-NhaB variants with Gly, Thr, or Glu at residue 147 showed a rapid $^{22}Na^+$ efflux (half-life of about 1.3 min; Fig. 6). Under these conditions, the cells carrying the Glu variant did not lose net Na^+ (data not shown), suggesting that all variants do not extrude net Na^+ under this condition. Since

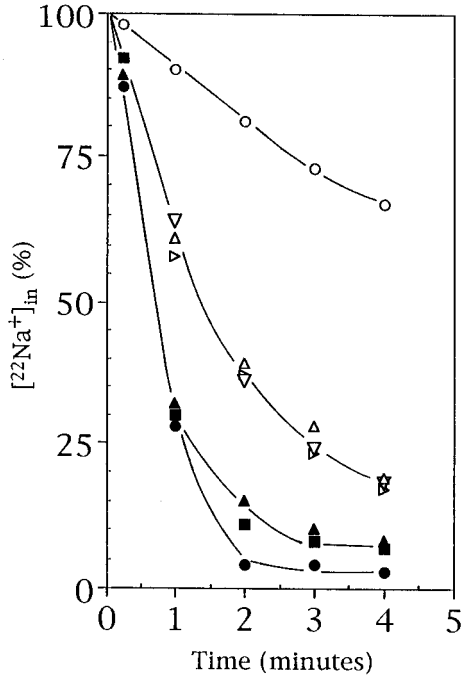

FIG. 6. $^{22}Na^+$ efflux from $^{22}Na^+$-equilibrated, Na^+-loaded TO114 cells. TO114 cells carrying pGEX-2TK (○), pGEX-*nhaB* (▲), pBD519 (■), pTN1 (●), pBD147G (△), pBD147T (▽), or pBD147E (▷) were loaded with $^{22}Na^+$. Subsequently, the cells were diluted in a medium without $^{22}Na^+$ and radioactivity remaining in the cells was measured as a function of time, as described in the text. The average values of at least three experiments are presented.

$^{22}Na^+$ efflux rates of all variants are about four times as high as those of control cells carrying vector plasmid (Fig. 6), we conclude that the residue-147 variants of Va-NhaB remain partially active in $^{22}Na^+$-Na^+ exchange activity under conditions at which they are inactive in Na^+-H^+ exchange activity.

One interpretation of our data is that Va-NhaB possesses different Na^+ and H^+ binding sites, as Ca^{2+}-ATPase has two Ca^{2+} binding sites (21). However, this is not the only interpretation. It is still possible that Na^+ and H^+ share a single translocation pathway but that the features that are required for binding the two different substrates are not identical. More experiments should be done to examine whether NhaB operates by a simultaneous mechanism that involves two pathways through the membrane or whether it operates by a sequential mechanism in a single translocation pathway, a so called "ping-pong" or "two-faced Janus" model. We are currently looking for amino acid residues whose mutation affects Na^+-Na^+ exchange activity.

We thank Evert P. Bakker for critical reading of the manuscript and for communicating his results to us prior to publication. We thank Kouich Sasaki and Manabu Kitamura for technical assistance.

This work was supported by the Terumo Life Science Foundation, the High-Tech Research Center of Meijo University, and a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture to T.N. (10672037) and N.U. (13206032 and 13660088) and by Center of Excellence (COE) research funding to N.U.

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