Characterization and Mapping of a Major Na^+/H^+ Antiporter Gene of *Escherichia coli*

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Using *in vivo* assays, we show that the Na^+/H^+ antiporter activity of the *Escherichia coli* mutant HIT-1 is reduced dramatically compared with activity in wild-type cells. An isogenic *nhaA* (formerly *antA*) deletion strain, however, is not significantly different from wild type in this respect. We call the locus affecting Na^+/H^+ antiporter activity of the HIT-1 mutant *nhaB*. The *nhaB* activity exhibits no pH dependence in the range between 7.0 and 8.5, whereas that of the *nhaA* gene increases considerably at pH levels above 8.0. Mutants with defects in *nhaB* grow normally on agar media containing 0.5 M NaCl, but *nhaA* mutants are sensitive to 0.5 M NaCl. We have mapped the *nhaB* mutation of HIT-1 to 25.6 min on the *E. coli* map. It is unlinked to the *nhaA* region, which is located at about 0.5 min. Since a cell with a mutation in *nhaB* alone is essentially Na^+/H^+ antiporter negative up to pH 8.0, we conclude that *nhaB* is required for the major Na^+/H^+ antiporter activity in the usual physiological pH range.

The Na^+/H^+ antiporter is a ubiquitous membrane carrier protein. It can utilize proton motive force to maintain an inwardly directed $[\text{Na}^+]$ gradient which can drive several Na^+ /substrate cotransporters. There are Na^+ cotransporters for substrates such as serine, proline, glutamate, and melibiose in a broad variety of bacteria. Since the extrusion of Na^+ is coupled to proton uptake, the Na^+/H^+ antiporter has the potential to acidify the cell interior. This is essential for cells in an alkaline environment (6). McMorrow et al. (9) showed that an *Escherichia coli* mutant lacking full Na^+/H^+ antiporter activity cannot grow at high pH. Niiya et al. (11) isolated a mutant (W3133-2S) which could cotransport melibiose with either Li^+ or Na^+ . They showed that this mutant has an altered melibiose permease and under certain conditions could be made Li^+ dependent (*melBLid*). It also had higher than normal Na^+/H^+ antiporter activity (*antup*). Goldberg et al. (1) separated the *antup* mutation from the *melBLid* mutation and mapped it to about 0.5 min on the *E. coli* chromosome. The *ant*⁺ region of the chromosome was inserted into a high-copy-number plasmid and cloned. Everted membrane vesicles made from such cells had greatly enhanced Na^+/H^+ antiporter activity. Karpel et al. (5) sequenced this region and identified an open reading frame of 1,085 bp as the putative *ant* gene that codes for Na^+/H^+ antiporter activity. Padan et al. (12) showed that a strain containing a deletion of this gene (Δant) gets progressively more sensitive to Na^+ (and Li^+) as the pH rises. Nevertheless, *E. coli* Δant grows normally up to pH 8.6 in the absence of added Na^+ . They also showed that the Δant mutation does not significantly affect Na^+/H^+ antiporter activity in everted vesicles. However, when vesicles are made from cells with a high-copy-number plasmid containing the *ant* gene, they exhibit an increasing Na^+/H^+ antiporter activity with increasing pH. Residual Na^+/H^+ antiporter activity of vesicles from the Δant strain is unaffected by pH changes and remains low (12). Ishikawa et al. (3) selected

another Na^+/H^+ antiporter mutant, HIT-1, which has virtually no Na^+/H^+ antiporter activity *in vivo* or *in vitro* at pH 8.0. Furthermore, this mutant fails to grow at high pH. The different features of the individual mutants with regard to Na^+ sensitivity and Na^+/H^+ antiporter activity imply that different genes are responsible for the variety of mutant phenotypes. The nomenclature has been changed to *nha* to relate these gene designations more uniquely to the Na^+/H^+ antiporter. For example, *antA* (12) has been changed to *nhaA*.

In this study, we investigate the Na^+/H^+ antiporter activity of HIT-1 and an *nhaA* deletion mutant and their combinations. To supplement previous *in vitro* work, the Na^+/H^+ antiporter activities of these cells were assayed *in vivo* by measuring the steady-state level of $[\text{Na}^+]$ in the medium, which is lowered when a Na^+ -cotransported substrate (serine or melibiose) is added to the medium. This change in external $[\text{Na}^+]$ reflects net antiporter-dependent accumulation of Na^+ in the cell (see Materials and Methods for details). The Na^+/H^+ antiporter-deficient cell HIT-1 was used to define and map a new gene, *nhaB*. We have localized the *nhaB* locus to 25.6 min on the *E. coli* map. HIT-1 cells accumulate Na^+ several hundredfold when measured at pH 7.0 to 8.0 (which reflects a very low level of antiporter activity), but the antiport is greatly increased at pH 8.5. Cells deleted for *nhaA*, on the other hand, show no pH dependence from pH 7.0 to 8.5. We conclude that *nhaB* is required for the major antiport activity which drives accumulated Na^+ out of the cell in the usual pH range (pH 7 to 8). The *nhaA* locus can enhance or substitute for it, to a large extent, at pH 8.5.

MATERIALS AND METHODS

Bacterial strains. The mutant bacterial strains DT5, HIT ΔAB^+ , HIT ΔAB^- , and HIT ΔAB^- (this report) and HIT-1 (3) are derivatives of W3133-2 (7), a prototrophic, streptomycin-resistant $\Delta lacZY$ derivative of *E. coli* K-12. The *recD* mutant V355 was obtained from G. C. Walker (14).

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Donor and transposon-containing strains used for mapping were obtained from Singer et al. (15).

Media. Cells were grown in L broth or in minimal medium AK (1) supplemented with 0.5% glycerol or, when required, 10 mM melibiose. Antibiotics for selections and screening were streptomycin (50 μ g/ml), tetracycline (25 μ g/ml), nalidixic acid (40 μ g/ml), and kanamycin (100 μ g/ml). Sodium sensitivity was screened for on L plates with 1.5% Difco agar and either 0.5 M NaCl or 0.5 M KCl (as a control).

Recombination systems. P1 transductions (10, 15), bacterial conjugations (10, 15), and linear plasmid transformations (4, 16) were carried out essentially as described previously.

Assay of Na^+/H^+ antiporter activity. The experimental setup for monitoring $[\text{Na}^+]$ in the medium consisted of a Na^+ combination electrode (Orion 84-11) and pH/voltmeter (Orion 720A) connected to a chart recorder (Kipp & Zonen) via a homemade operational amplifier device for offsetting the electrode readings into the low-sensitivity ranges (1 to 20 mV) of the chart recorder. All measurements were carried out in plastic containers at 37°C, stirred by a water-driven magnetic stirrer. Cells were grown to log phase (optical density at 600 nm = 0.4), washed three times in 0.1 M morpholinopropanesulfonic acid (MOPS; pH 7.0), and resuspended in 0.1 M Tricine (pH 7.0 to 8.5)–50 μ M NaCl to a cell density of 2.7×10^9 /ml. The Na^+ electrode was submerged in 4.2 ml of this suspension. When the potential (reflecting $[\text{Na}^+]$) stabilized, we added a Na^+ -cotransported substrate (serine or melibiose) in a small volume to a final concentration of 2 mM. When the $[\text{Na}^+]$ signal restabilized, the signal was titrated back to its origin with 1- μ l NaCl solutions (1 or 10 mM) to standardize the $[\text{Na}^+]$ scale. When the Na^+/H^+ antiporter activity is high, the final, steady-state level of Na^+ in the medium is not much lower than the 50 μ M of the original medium. This is because the cotransported Na^+ inside is exchanged rapidly for H^+ outside. The lower the antiporter activity, the further the steady-state $[\text{Na}^+]$ rises in the cell and decreases in the medium.

RESULTS

Separation of Na^+ sensitivity from Na^+/H^+ antiporter activity. Padan et al. (12) found that everted vesicles of *nhaA* deletion mutants possess wild-type levels of Na^+/H^+ antiporter activity. This finding suggested that the *nhaA* locus is not the only, or even the major, gene for Na^+/H^+ antiporter activity. Another mutant selected by Ishikawa et al. (3), in contrast to *nhaA* deletion strains such as NM81 of Padan et al. (12), shows very little or no Na^+/H^+ antiporter activity compared with wild-type cells either in vivo (Fig. 1) or in vitro (3). Thus, it would seem that a mutation in another gene was responsible for the drastically reduced Na^+/H^+ antiporter activity in the HIT-1 mutant. We suspected that HIT-1 is mutant in a gene, *nhaB*, separate from *nhaA* which affects Na^+/H^+ antiport.

To investigate the features of both *nhaA* and *nhaB* individually and together, we studied the growth properties and Na^+/H^+ antiporter activities of both singly and doubly mutant cells. We made our *nhaA* deletion mutants, DT4 and DT5, by removing the complete *nhaA* gene from the chromosome and replacing it with a kanamycin cassette (Fig. 2). Table 1 shows that the growth of our *nhaA* deletion mutant, DT5, like that of NM81 of Padan et al. (12), was sensitive to high $[\text{Na}^+]$ in the media. Wild-type *nhaA*⁺ cells were not. The Na^+/H^+ antiporter activity of DT5 at pH 8.0, however, is not significantly different from that of wild-type cells (Fig. 1A). Table 1 (line 3) also shows that Na^+ resistance can be

FIG. 1. In vivo measurements of Na^+/H^+ antiporter activity. Shown are two typical Na^+ electrode readings in cell suspensions prepared as described in Materials and Methods. As soon as the electrode reading stabilized, a serine or melibiose solution was added. The decrease in $[\text{Na}^+]$ was monitored until a new steady state was reached and titrated back to its origin by Na^+ standards. (A) Wild-type W3133-2 cells; (B) HIT-1 mutant cells.

restored by transduction to *nhaA*⁺ (using a closely linked Tn10 for selection). Therefore, *nhaA* is required for growth at high $[\text{Na}^+]$, whereas very high Na^+/H^+ antiporter activity is still observed in the absence of *nhaA*. Since the mutant HIT-1 has little or no Na^+/H^+ antiporter activity at pH 8.0 (Fig. 1B), and Na^+ sensitivity was explained as a consequence of the loss of Na^+/H^+ antiporter activity provided by *nhaA* (12), we wondered whether HIT-1 was sensitive to high $[\text{Na}^+]$ in the medium. Table 1 (line 4) shows that HIT-1 can grow in high $[\text{Na}^+]$ even though it exhibits little or no

FIG. 2. Construction of an *nhaA* deletion strain, DT4. The *nhaA* gene (and small flanking regions) on a plasmid was deleted and replaced by a kanamycin cassette. The *nhaA* gene in the cell was replaced by the kanamycin cassette by homologous recombination of the linearized plasmid. To facilitate this event, a *recD* mutant strain V355 (14) was transformed to Kan^r by a linear *XmnI* fragment of plasmid pTP3. In pTP3, the 3.1-kb *NsiI*-*PstI* fragment of pGM69 (1, 8) including the *nhaA* gene was replaced by the kanamycin cassette from pRI133 (2), which was excised by *PstI* cleavage. The *TaqI* site in pTP3 was formed from the *NsiI*-*PstI* ligation. The *XmnI* digest of pTP3 served to linearize the plasmid and to remove its *ori* region. After transformation of the *recD* strain V355 to Kan^r by the linear fragment, a P1 lysate from this strain (DT4) was used to transduce W3133-2 and the *nhaA* deletion strain (DT5) was selected from the infected culture by kanamycin resistance.

TABLE 1. Comparison of Na⁺/H⁺ antiporter activity and salt sensitivity

Strain	Antiporter activity	Growth on L medium with:	
		0.5 M Na ⁺	0.5 M K ⁺
1. W3133-2 (wild type)	+	+	+
2. DT5	+	—	+
3. DT5 × P1 <i>nhaA</i> ⁺ <i>tet</i> ^{ra}	+	+	+
4. HIT-1	—	+	+
5. HIT-1 × P1 <i>nhaA</i> ⁺ <i>tet</i> ^r	—	+	+
6. HIT-1 × P1 <i>nhaA::kan</i> ^r (HITΔA)	—	—	+

^a In this strain, the linkage of *nhaA* to *tet* is 75%.

Na⁺/H⁺ antiporter activity in vivo. This is also true when the cell has been transduced to Tet^r (Table 1, line 5) by using the same Tn10 linked to *nhaA*⁺ as for DT5 above. However, introduction of a Δ *nhaA* into HIT-1 by P1 transduction (Table 1, line 6) made this cell Na⁺ sensitive while its Na⁺/H⁺ antiporter remained inactive. Therefore, the mutation in HIT-1 which is responsible for the loss of Na⁺/H⁺ antiporter activity is independent of *nhaA*. The sensitivity of cell growth to high [Na⁺] depends on *nhaA* and is independent of the *nhaB* mutation which dramatically reduces Na⁺/H⁺ antiporter activity measured in vivo at pH 8.0. The finding that HIT-1 has a mutation in an Na⁺/H⁺ antiporter gene which is different from *nhaA* made this mutant an ideal tool for mapping this apparently important Na⁺/H⁺ antiporter gene.

Mapping of *nhaB*. For the initial stage of mapping, we crossed HIT-1 recipients with a number of marked (Tet^r or Kan^r) donor strains (15). When HIT-1 was mated with donor strains with Tet^r markers covering the region of 25 to 50 min of the *E. coli* map, there were some Tet^r exconjugants which elicited a wild-type response (Fig. 1A) with regard to Na⁺/H⁺ antiporter activity.

After localizing *nhaB* to the region of 20 to 27 min by interrupted matings, we used the set of *kan* and *tet* insertions in this region of the wild-type chromosome (15) to map the *nhaB* gene more precisely by P1 transduction using HIT-1 as a recipient (Fig. 3). By looking at the Na⁺/H⁺ antiporter phenotype of antibiotic-resistant transductants from P1 ly-

sates of 18 different *tet*-marked strains from 17.75 to 39.5 min, we found that the *nhaB*⁺ gene was cotransduced with only two markers: at 25.25 min (frequency = 0.35, *n* = 14) and at 25.75 min (frequency = 0.43, *n* = 23). No *nhaB*⁺ cotransduction by P1 was found with any of the other 16 markers (*n* = 10 for each marker). Finally, the mapping position of *nhaB* was further determined with a set of donor strains with vicinal Tet^r and Kan^r markers made from the P1 mapping set of Singer et al. (15), which enabled us to transduce defined regions of chromosomal DNA flanked by the two markers. Doubly marked P1 donor strains were constructed by inserting the additional marker into a given P1 donor strain 0.5 to 1.0 min apart from the existing marker, and then selection for both markers was performed. When we selected simultaneously for two different markers (Kan^r and Tet^r), at 25.25 and 25.75 min, 100% of the transductants (*n* = 8) had normal levels of Na⁺/H⁺ antiporter activity restored in the formerly antiporter-negative strain HIT-1 (Fig. 3). This localizes *nhaB* to that region. To prove that *nhaB* was between these two markers and not outside of them, we selected simultaneously for markers at 24.25 and 25.25 min (*n* = 9) or at 25.75 and 26.75 min (*n* = 9). By testing for Na⁺/H⁺ antiporter in the transductants, we found cotransduction frequencies of 0.33 and 0.56, respectively. If *nhaB* had been outside of the two central markers, the cotransduction to the side with the gene would have had to give a frequency of 1, whereas cotransduction to the other side would have given a frequency of about 0. The actual cotransduction values of 0.33 to the left and 0.56 to the right suggest a location of about 25.6 min.

Dependence of *nhaA* and *nhaB* Na⁺/H⁺ antiporter activity on medium pH. We were now able to create an isogenic set of the four combinations containing the *nhaA* and *nhaB* loci to investigate the pH dependence of their Na⁺/H⁺ antiporter activities in vivo. We started with HIT-1 and HITΔA (Table 1), which we henceforth denote as HITA⁺B[−] and HITΔAB[−], respectively. The mapping experiments yielded an antiporter-positive transductant of HIT-1, HITA⁺B⁺. Finally, we transduced HITA⁺B⁺ with P1.DT5 to delete *nhaA* from this strain to obtain HITΔAB⁺, which, like DT5, is Na⁺ sensitive (data not shown). Furthermore, to study the phenomenon of excess *nhaA* (1, 12) in a cell without interference of *nhaB*, HITA⁺B[−] was transformed to Amp^r by pGM36 a multicopy plasmid containing the *nhaA* region.

The pH dependence of Na⁺/H⁺ antiport in this set of strains was measured as described for Fig. 1, which shows the relative level of Na⁺/H⁺ antiporter activity assayed by measuring the decrease of [Na⁺] in the medium, which reflects antiporter-dependent accumulation of Na⁺/substrate-cotransported Na⁺ in the cell. Thus, the higher the Na⁺ concentration remaining in the medium the more pronounced the Na⁺/H⁺ antiporter activity. We prefer this in vivo assay to the potential ambiguities of in vitro measurements on everted membrane vesicles in which the cytoplasmic milieu (e.g., pH) of the antiporter and the topology of the membrane are altered.

Figure 4A shows the Na⁺/H⁺ antiporter activity of strains deleted for the *nhaA* gene at pH values from 7.0 to 8.5. As seen in the upper curve, HITΔAB⁺ has a very high Na⁺/H⁺ antiporter activity with no pH dependence in the range investigated here. This Na⁺/H⁺ antiporter activity is severely reduced in HITΔAB[−], which has neither of the two antiporter systems. Thus, the cotransporter-mediated net Na⁺ uptake is probably limited only by the reduction of the effective concentration gradient of Na⁺ and melibiose. These experiments indicate that *nhaB* alone, in the absence

FIG. 3. Mapping of *nhaB* by P1 transduction. The *nhaB* mutant strain HIT-1 was transduced by P1 grown on various mapping strains (15), using *tet* and *kan* markers for selection. Transductants were tested by screening for wild-type Na⁺/H⁺ antiporter activity in physiological measurements (compare with Fig. 1). Frequencies of cotransduction of the individual antibiotic resistance selection marker(s) with *nhaB* are given in parentheses above the map position (in minutes) (15) for single-marker selection and below the lines between the marked map positions for double-antibiotic-marker selection (see text for more details). The lower arrow points to the locus of the transposon in the *fadR* gene; the upper arrow points to the approximate locus of the *nhaB* mutation.

FIG. 4. pH dependence of Na⁺/H⁺ antiporter activity in an isogenic set of Na⁺/H⁺ antiporter mutants. Na⁺/H⁺ antiporter activity was calculated from Na⁺ electrode readings as described for Fig. 1 at various pHs. Readings were taken at equilibrium before and after addition of 2 mM melibiose to the medium. Melibiose cotransports Na⁺ into the cells, thus reducing the external [Na⁺] from the initial 50 μM to a lower value depending on the Na⁺/H⁺ antiporter activity of the cells. Δ, HITΔAB⁺; ○, HITΔAB⁻; ▲, HITA⁺B⁺; ●, HITA⁺B⁻; ■, HITA⁺B⁻/pGM36.

of *nhaA*, provides most of the Na⁺/H⁺ antiporter activity in this physiological pH range. We also show that the Na⁺/H⁺ substrate cotransport involved in this assay of Na⁺/H⁺ antiporter activity exhibits no pH dependence of its own (Fig. 4A, HITΔAB⁻) in the pH range studied. This is a prerequisite for the experiments shown in Fig. 4B. For HITA⁺B⁺, when both antiporter genes are present (Fig. 4B), a very slight pH-dependent increase of antiporter activity is observed. The experiments with HITA⁺B⁻ demonstrate that over a pH range from 7.0 to 8.0 there is little or no participation of *nhaA*⁺, but a sharp increase of *nhaA* activity becomes evident above pH 8.0. However, even at pH 8.5 it does not reach the level of *nhaB* (compare with Fig. 4A). When *nhaA* is present in high copy number (on plasmid pGM36), it can provide considerable Na⁺/H⁺ antiporter activity even at pH 7.0. This level increases to almost wild-type levels at pH 8.5 (Fig. 4B). Thus, in high copy number and at high pH, *nhaA* can partially compensate for a lack of *nhaB* activity.

DISCUSSION

The *nhaB* gene is part of a Na⁺/H⁺ antiporter system of *E. coli* and is unlinked to *nhaA*. In this work, we mapped the *nhaB* mutation to the vicinity of 25.6 min and showed that *nhaB* is required for most of the Na⁺/H⁺ antiporter activity in *E. coli* from pH 7.0 to 8.5. We do not yet know whether *nhaB* codes for a structural or for a regulatory protein. Deletions of the *nhaA* gene (12; this report) demonstrate that the *nhaB* Na⁺/H⁺ antiporter system can work in the absence of *nhaA* both in vitro and in vivo. The clearest phenotype of *nhaA* expression in vivo is the inability of *nhaA*-deleted cells to grow at high concentrations of Na⁺. There is very little or no Na⁺/H⁺ antiporter activity measurable in such cells from pH 7.0 to 8.0. Only at pH values above 8.0 can a significant Na⁺/H⁺ antiporter activity be observed from *nhaA*⁺ cells. This activity is most readily observed when *nhaB* is mutant. Increased *nhaA* Na⁺/H⁺ antiporter activity can also be seen in *antup* mutants (11) and in strains carrying multicopy plasmids with wild-type inserts from the *antup* locale at 0.5 min (1). It is not yet clear whether the enhancement of Na⁺/H⁺ antiporter activity in the cell by excess *nhaA*⁺ or by

high pH is due to an independent Na⁺/H⁺ antiporter system or to participation of *nhaA* in the *nhaB* system in a structural or regulatory role.

Padan et al. (12) demonstrated a pH-dependent *nhaA* Na⁺/H⁺ antiporter system, which we have verified here. However, the high *nhaB* activity which we see in vivo is not seen in their in vitro experiments. Thus, the in vitro measurements of *nhaA* in high copy number, in the presence of *nhaB* (12), resemble our results of *nhaB*-negative HITA⁺B⁻/pGM36. Furthermore, their measurements using the *nhaA* deletion mutant resemble those for our double mutant, HITΔAB⁻. We think it possible that in their preparation of everted membrane vesicles, *nhaB* activity is lower than in vivo levels. This may explain the quantitative discrepancies between our findings and those of Padan et al. (12).

The mapping of the *nhaB* locus should facilitate cloning of *nhaB*. This, in turn, should make it possible to sequence and to delete this gene. The sequence of the *nhaB* protein might provide insights into homology relationships between *nhaB* and other Na⁺/H⁺ antiporter systems. Our analysis of the *nhaA* sequence revealed no clearly significant homology to other proteins in the EMBL data bank, including the eucaryotic Na⁺/H⁺ antiporter (13).

A deletion of *nhaB* should help to classify under what conditions the *nhaA* system may function independently of the *nhaB* system and to investigate the possible interactions of the proteins associated with these two genes, especially with respect to the Na⁺ sensitivity and the putative Na⁺ leak. Padan et al. (12) postulated a pH-dependent Na⁺ leak and suggested that the *nhaA* Na⁺/H⁺ antiporter activity could compensate for it. However, cells deleted for *nhaA* are Na⁺ sensitive but are otherwise only slightly affected, since the bulk Na⁺/H⁺ antiporter activity in vivo derives from *nhaB*. The *nhaA*-deficient cells remain viable even at high pH and become inviable only in the presence of high [Na⁺] (12). Thus, an *nhaB* deletion may help to determine whether the *nhaA* protein compensates for a lack of sufficient Na⁺/H⁺ antiporter activity required in high [Na⁺] or reduces a Na⁺ leak physically, for example, by interaction with *nhaB* or some other Na⁺ pore.

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