Deletion of ant in *Escherichia coli* Reveals Its Function in Adaptation to High Salinity and an Alternative Na\(^+\)/H\(^+\) Antiporter System(s)*

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We have deleted the chromosomal ant gene from *Escherichia coli* by substitution with the kan gene, which encodes kanamycin resistance. The Δant strains obtained cannot adapt to high sodium concentrations (700 mM, pH 6.8), which do not affect the wild type. The Na\(^+\) sensitivity of Δant is pH dependent, increasing at alkaline pH. Thus at pH 8.5, 100 mM NaCl retard growth of Δant with no effect on the wild type. The Δant strains also cannot tolerate the toxic effects of Li\(^+\) ions, a substrate of the Na\(^+\)/H\(^+\) antiporter system. However, growth of these strains is normal on carbon sources which require Na\(^+\) ions for transport and growth. Moreover, antiporter activity, as measured in everted membrane vesicles, is not significantly impaired.

A detailed analysis of the remaining antiporter activity in a Δant strain reveals kinetic properties which differ from those displayed by the ant protein: (a) \(K_m\) for transport of Li\(^+\) ions is about 15 times higher and (b) the activity is practically independent of intracellular pH. Our results demonstrate the presence of an alternative Na\(^+\)/H\(^+\) antiporter(s) in *E. coli*, additional to ant system.

All growing cells extrude sodium ions actively and maintain a sodium concentration gradient directed inward (1–5). Maintenance of low intracellular concentration of Na\(^+\) seems a necessity in all living cells and is ascribed to inhibitory effect of high Na\(^+\) to essential enzymes. The Na\(^+\) gradient serves as a driving force for transport systems catalyzing sodium/substrate symport (2, 3, 5). Sodium circulation has also been implicated in regulation of intracellular pH (6) and energy homeostasis (7, 8).

In bacteria generation of a Na\(^+\) gradient is often attributed to the Na\(^+\)/H\(^+\) antiporter activity which is most widely distributed and is energized by the proton electrochemical gradient (9, 10). Three types of primary pumps extruding Na\(^+\) have also been described (for review see Ref. 11). The Na\(^+\)/H\(^+\) antiporter activity has been thoroughly studied in intact cells and isolated membrane vesicles (10). It is electrogenic with an overall stoichiometry of Na\(^+\)/H\(^+\) of above 1 (8, 10, 12), and its activity is regulated by intracellular pH (10). However, although the activity has been reconstituted in proteoliposomes the protein(s) involved has not been purified (13, 14).

A genetic approach has been undertaken to study the Na\(^+\)/H\(^+\) antiporter system in *Escherichia coli*. Different mutants have been isolated which lost the Na\(^+\)/H\(^+\) antiporter activity as well as the capacity to regulate intracellular pH at alkaline pH, implying the role of the antiporter in pH homeostasis of bacteria (15–17). None of these mutations have been mapped to a structural gene coding for antiporter activity.

Tsuiya, Wilson and colleagues (18) isolated a mutant in *E. coli* that shows increased rather than decreased antiporter activity in isolated membranes. These cells also tolerate much higher Li\(^+\) concentrations (100 mM) as compared with the wild type cells (10 mM). The Li\(^+\) resistance has been ascribed to the excretion of the ion by the enhanced antiporter. This mutation designated antup has been mapped at about 0.5 min from the wild type gene ant cloned in plasmid pBR322 (19, 20). When in high copy number (plasmidic + chromosomal) the wild type ant confers Li\(^+\) resistance to cells and increases Na\(^+\)/H\(^+\) antiporter activity in membranes (19) i.e. the antup phenotype. The ant gene has been sequenced and found to encode a membrane protein of \(M, 38,000\) (20). The protein has been purified and reconstituted in proteoliposomes in a functional form.

In the present work we studied the role of ant in wild type cells and inquired whether Na\(^+\)/H\(^+\) antiporter activity(s), other than ant, exists in *E. coli*. For these purposes we have deleted ant from the chromosome and studied the phenotype of the Δant strain pertaining to growth tolerance to Na\(^+\), Li\(^+\), and pH and the Na\(^+\)/H\(^+\) antiporter activity in isolated membrane vesicles.

**MATERIALS AND METHODS**

Bacterial Strains, Plasmids, and Culture Conditions—Bacterial strains used in this study are K12 derivatives. TA15 is melBlidant ΔlacZY (19). JC7623 is recB21 recC22abB15th (21). Cells were grown in L broth or in L broth of which NaCl was replaced by KCl (87 mM, LBK) or minimal medium A (22) without sodium citrate, supplemented with thiamine (1 μg/ml), 0.5% of glycogen or 10 mM melibiose as required. For JC7623 and its derivatives threonine (100 μg/ml) was also added. Growth at pH 8.6 was conducted in 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. Where indicated Na\(^+\) and/or K\(^+\) concentrations were changed and Li\(^+\) added. For plates 1.5% Difco agar was added. For selection of plasmids 100 μg/ml ampicillin or 50 μg/ml kanamycin were used.

Construction of plasmids are shown in Fig. 1.

Transduction was carried out with Ploor phage as described (23).

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2 The abbreviations used are: CAPS, 3-(cyclohexylamino)propane-sulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; kb, kilo base pairs; Hpes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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Protein concentration in cells and everted membrane vesicles was estimated as based on its ability to collapse a transmembrane pH gradient. Acridine fluorescence was monitored to estimate ΔpH as previously described (14, 19). Fluorescence of acridine orange was monitored in a Perkin-Elmer fluorimeter (Luminescence Spectrometer, LS-5). Exciting light was 430 nm and emission light was measured at 570 nm.

Na⁺/H⁺ antiporter activity in everted membrane vesicles was estimated based on its ability to collapse a transmembrane pH gradient. Acridine fluorescence was monitored to estimate ΔpH as previously described (14, 19). Fluorescence of acridine orange was monitored in a Perkin-Elmer fluorimeter (Luminescence Spectrometer, LS-5). Exciting light was 430 nm and emission light was measured at 570 nm.

RESULTS

To elucidate the role of ant gene in E. coli cells, it was essential to disrupt the chromosomal ant and to obtain cells devoid of an active gene. For this purpose we have constructed plasmids pDTK1, pDTK2, and pKR36 (Fig. 1) and produced deletion and insertion mutations in the wild type ant gene of E. coli chromosome. To obtain pDTK1 about ⅔ of the ant sequences of pGM36 (19) have been substituted with the gene encoding for kanamycin resistance. The C- and N-terminal regions of ant and additional chromosomal flanking regions of 0.8 and 1.4 kb, on each side, respectively, were left unmodified. pDTK2 is identical to pDTK1 except for the orientation of the kanamycin gene which is reversed. Almost all ant sequences were deleted from pGM36 to obtain pKR36 leaving only the 22 base pairs of the N-terminal.

When pGM36 is transformed to TA15 cells it confers the antup phenotype upon the cells, i.e. they become Li⁺ (100 mM) resistant and their membranes exhibit enhanced Na⁺/H⁺ antiporter activity (19). TA15 cells transformed with either pDTK1, pDTK2, or pKR36 remained Li⁺ sensitive like the parent strain and did not show enhanced Na⁺/H⁺ antiporter activity in their membranes (not shown). These results verify that the ant gene is inactive in the plasmids bearing the disrupted ant genes.

To obtain cells without a functional ant, the disrupted ant genes were exchanged with the chromosomal wild type ant of E. coli by homologous recombination. Plasmids pDTK1, pDTK2, and pKR36 were linearized by digestion with PvuII 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 (21).

Based on the functions attributed to the Na⁺/H⁺ antiporter (see Introduction and Refs. 2-6) it was expected that recombinants carrying the disrupted ant will be sensitive to Na⁺ and prefer neutral pH and rich medium. Therefore, transformants of linearized pDTK1, pDTK2, or pKR36 were plated on LBK, pH 6.8, agar plates containing kanamycin. Recombinants originated by double crossing over (Fig. 2) were obtained by growth on kanamycin and scoring for sensitivity to ampicillin, the vector's selective marker. Hence, Kan² Amp² recombinants were expected to contain, instead of the wild type ant, the deleted ant of pDTK1, pDTK2, or pKR36 with the insertion of the kanamycin gene. One of each type of recombinants designated NM8, NM9, and JCK2, respectively, were used for further study.

To verify that the recombinants contain the deletion and insertion of the ant gene, we looked for the presence of different DNA sequences by hybridization to the DNA of NM8 as compared with the DNA of the wild type. When the BamHI-BamHI DNA fragment of pUC71K containing the kanamycin gene (Fig. 1) was used as a DNA probe, hybridization was observed with the DNA of NM8 but not with the wild type DNA (Fig. 3A). As expected for the distances between the restriction sites of NM8 DNA (Fig. 2) only one BamHI-BamHI fragment of NM8 DNA hybridized, and it was of 2.5 kb. On the other hand two HindIII-Hind111 fragments of 1.4 and 3.3 kb hybridized (Fig. 3A).

When the MluI-BglII DNA fragment of pGM36 which was deleted from ant in NM8 (Figs. 1 and 2) served as a probe there was hybridization only with the wild type DNA while the deletion mutant DNA did not show any homology to the probe (Fig. 3B). As expected after restriction with BamHI or HindIII (Fig. 2), the wild type DNA fragments which hybridized were 1.85 and 5.2 kb or 4.1 kb, respectively.

Utilizing the AuaI-BstXI fragment of pGM36 which bears the entire ant gene (Figs. 1 and 2) as a probe both the wild type DNA and NM8 DNA exhibit hybridization albeit the size of the DNA fragments were different and accorded the changes in the restriction map of the two strains DNA. The BamHI-BamHI and the HindIII-HindIII fragments of the wild type which hybridized were the same as with the previous...
FIG. 2. Construction of an *E. coli* mutant carrying a deletion and insertion in ant. Plasmid pDTK1 was linearized with *Pvu*I and transformed in JC7623 which allows recombination between the linearized plasmid and the chromosome but does not allow propagation of plasmids. (For details see text). A scheme of the double crossing over event leading to the deletion insertion mutant is depicted. This mutant was selected as Kan^R^Amp^S^ as opposed to a single crossing over product which is Kan^R^Amp^S^. Genes and flanking regions are marked as in Fig. 1. Thin arrows describe distances with the number of kb below them.

![Diagram of DNA restriction endonuclease digests](image)

**A.** 2 3 4

**B.** 2 3 4

**C.** 2 3 4

WT M WT M WT

HindIII BamHI

HindIII BamHI

M WT M WT M

BamHI HindIII

BamHI HindIII

WT DNA

Mutant DNA

FIG. 3. Southern blot of restriction endonuclease digest of the DNA of the mutant (NM8) and wild type (TA15) hybridized to different probes. Southern blots and DNA-DNA hybridization were obtained as described under "Materials and Methods." The following different probes were used. A, the BamHI-BamHI 1.2-kb fragment excised from pUC71K and containing the kanamycin gene; B, the *BglII*-MluI 1.1-kb fragment of ant obtained from pGM36; C, the *AvaI*-BstXI 1.38-kb fragment bearing the entire ant derived from pGM36. Chromosomal DNA of TA15 (WT) and NM8 (M) were digested with BamHI or HindIII as depicted. Fragment sizes are given in kb.

A, B, C

![Southern blot diagram](image)

probe (compare Fig. 3, C and B). On the other hand the NM8 mutant hybridizing fragments after BamHI or HindIII restriction were of 2.5 and 5.1 kb or 3.3 and 1.42 kb, respectively. We therefore conclude that NM8 indeed harbors the deletion and the kanamycin insertion in its ant gene. These results also show that ant is present in only one copy in the wild type chromosome.

TA15 is the *E. coli* strain which we have used for all physiological studies of ant. Therefore, for the study of the effect of mutations in ant on the physiology of the cell we have transferred the deletion/insertion mutations to TA15 by P1 transduction and obtained strain NM81 and NM91 from NM8 and NM9, respectively, and strain RK20 from JCK2. In accordance with the map position of ant (19), the linkage between *thr* and the ant mutation as selected by kanamycin was confirmed in all transductions. The hybridization experiments conducted with NM8 (Fig. 3) were repeated with NM81 and RK20 and in all cases the deletion and insertion were confirmed (not shown).

It is anticipated that a deletion/insertion mutation in the ant gene will reveal ant functions. It will grow only under conditions compensating for and therefore reflecting ant activities. The growth of NM81 on minimal medium A with no Na^+^ added and with either melibiose or glycerol (not shown) as carbon sources was identical to that of the wild type-TA15 (Fig. 4A). Although not added, this medium contains a contamination of 0.5–1 mM Na^+^ (31). As shown before addition of 100 mM Li^+^ but not Na^+^ or K^+^ markedly decreased the growth of TA15 (19). However, growth of NM81 was more sensitive to Li^+^ with doubling time of 7 h as opposed to 3.9 h of TA15 (Table I). Addition of 10 mM Li^+^ already retarded growth of NM81 but not of TA15 (not shown). This supersensitivity of NM81 is due to lack of functional ant since NM81 cells transformed with pGM36, high copy number plasmid bearing ant, became resistant to Li^+^ as previously documented for TA15 cells transformed with this plasmid (19). The transformed cells grow in the presence of Li^+^ (100 mM) as well as without it (Table I). These results confirm our previous suggestion that ant confers Li^+^ resistance.

The growth of NM81, NM91, and RK20 on LB broth, pH 6.8, of which Na^+^ has been replaced by K^+^ (LBK) was identical to that of the wild type (not shown). Whereas the wild type retained identical growth capacity up to at least 0.7 M
Multiple Na⁺/H⁺ Antiporters in E. coli and Adaptation to Salinity

TABLE I

<table>
<thead>
<tr>
<th>Medium ion conc.</th>
<th>Medium A, melibiose</th>
<th>LB (pH 6.8)</th>
<th>LB (pH 7.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>No addition</td>
<td>Li⁺ (0.1 M)</td>
<td>Na⁺ (0.08 M)</td>
</tr>
<tr>
<td>TA15</td>
<td>1.1</td>
<td>3.9</td>
<td>0.7</td>
</tr>
<tr>
<td>NM81</td>
<td>1.1</td>
<td>7.0</td>
<td>1.1</td>
</tr>
<tr>
<td>NM81/pGM36</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

NaCl, increasing NaCl concentration led to decrease in the growth of the mutants, and at 0.7 M NaCl it was practically arrested (Table I). The sensitivity of NM81 to Na⁺ was clearly due to lack of functional ant. It was not caused by the presence of the kanamycin insertion since the mutant NM82, with the reversed kan gene, was sensitive to Na⁺ to the same degree, both in the presence and absence of kanamycin (not shown). Furthermore, NM81 transformed with pGM36, which carries an intact ant gene, regained the wild type resistance to Na⁺ (Table I).

The Na⁺ sensitivity of the mutants could be due to the nonspecific effect of increased ionic strength and/or increased osmolality. These possibilities were ruled out since when 0.7 M KCl rather than NaCl were added to the LBK medium growth of NM81 was identical to that of the wild type or to NM81/pGM36 (not shown). The Na⁺ sensitivity could also stem from lack of enough K⁺ under the conditions of high Na⁺. This alternative was also ruled out since addition of up to 10 mM KCl did not relieve the inhibition caused by NaCl (not shown). Hence, high Na⁺ has a specific inhibitory effect on the mutant lacking intact ant. Taken together we conclude that functional ant gene is required for the adaptation of E. coli to high salinity as well as for enduring Li⁺.

Changing the pH of LBK to 7.5 had no effect on growth of NM81 nor on that of TA15 (not shown) even when KCl concentrations increased to 0.5 M (Table I). However, in marked contrast to TA15, whose growth was not significantly affected by addition of NaCl, the Na⁺ sensitivity of NM81 increased at pH 7.5 (Table I). Whereas at pH 6.8, 0.7 M NaCl were required to inhibit growth of NM81, at pH 7.5, similar inhibitory effects were obtained already by addition of 0.5 M NaCl. To further study the effect of pH, NM81 and TA15 were grown in MTC medium at pH 8.6. Their doubling time was identical (1.5 h) with either glycerol or melibiose as carbon sources. However, addition of only 0.1 M NaCl, but not 0.1 M KCl (not shown) inhibited growth of NM81 (doubling time of 4.5 h). Growth of TA15 was not affected. Hence, although NM81 is not sensitive to pH its Na⁺ sensitivity is markedly influenced by pH.

It has previously been shown that ant affects the Na⁺/H⁺ antiporter activity of isolated membrane vesicles (19, 20). Cells bearing the mutation antup in the ant locus or transformed with high copy number plasmid bearing ant display increased Na⁺/H⁺ antiporter activity and Li⁺ resistance. These results suggested that the increased antiporter activity is the basis of the resistance. Therefore, we measured the Na⁺/H⁺ antiporter activity in membrane vesicles isolated from NM81 and TA15.

Surprisingly, membrane vesicles from NM81, (the Δant strain) displayed Na⁺/H⁺ and Li⁺/H⁺ antiporter activities that were only 40—50% lower than that displayed by membranes from TA15 (Fig. 4, A and B). This finding was observed regardless of whether the cells were grown in minimal medium or in LBK, although in the latter case all signals were proportionally higher (compare Fig. 4 with 5). It is evident, therefore, that in spite of the deleted ant, NM81 possesses a Na⁺/H⁺ and a Li⁺/H⁺ activity. It also grows in minimal medium with melibiose as a carbon source, and this growth is Na⁺ dependent (not shown). These results imply that the antiporter activity observed in NM81 is sufficient to maintain a Na⁺ gradient and support melibiose transport.

A K⁺/H⁺ antiporter activity has previously been documented in membrane vesicles isolated from E. coli cells (32, 33). In contrast with the Na⁺/H⁺ antiporter activity which is specific to Na⁺, this system has a low ion specificity and exchanges H⁺ for both Na⁺ and K⁺. In addition the K⁺/H⁺ antiporter system is very sensitive to digestion by trypsin whereas the Na⁺/H⁺ antiporter is resistant to such treatment (33). Since all measurements summarized in Fig. 4 were performed at K⁺ concentrations (150 mM) saturating the K⁺/H⁺ antiporter (Keq, 3—7.5 mM (33)), we assumed that under these conditions the K⁺/H⁺ antiporter does not play a major role in the Na⁺/H⁺ antiporter activity of any of these membranes. To further verify this assumption, we measured the K⁺/H⁺ antiporter activity in membrane vesicles isolated both from TA15 and NM81. When measured in the presence of choline chloride rather than KCl, in both membranes similar K⁺/H⁺ antiporter activity was monitored (Fig. 5). Furthermore in both membranes treatment by trypsin markedly decreased the K⁺/H⁺ antiporter activity with almost no effect on the Na⁺/H⁺ antiporter activity (see for example Fig. 5C). These results imply that the activity of TA15, the Na⁺/H⁺ antiporter activity activity of isolated membrane vesicles derived from TA10 and NM81. Everted membrane vesicles were prepared from TA15 (a), and NM81 (b and c) grown in minimal medium A with glycerol as a carbon source. The experimental system and protocol was that described in Fig. 4 but the reaction mixture contained 10 mM Tris-Hepes, pH 8.0, 140 mM choline chloride, 5 mM MgCl₂, and 100 μg of protein. When indicated KCl or NaCl were added to 8 mM concentrations. Trypsin treatment was carried out according to Ref. 33. Trypsin (Sigma, 15 μg/ml) was added to the reaction mixture containing the membranes and incubation continued for 3 min. Then the trypsin inhibitor (Sigma) was added (75 μg/ml), and 30 s later lactate was added to onset the reaction.
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H+ antiporter activity observed in NM81 is not related to the K+/H+ antiporter system and therefore represents an as yet unknown system(s), additional to ant system. Since ant is disrupted in NM81, the activity observed in NM81 membranes clearly represent a second system(s) other than ant. A mixture of the two types, most probably, comprises the activity observed in membrane vesicles of TA15, the wild type strain. However, TA15 transformed with pGM12, a high copy number plasmid bearing ant, may yield membranes representing mainly the ant system. In these membranes minimal interference is expected from the second system since it is most probably present in a single copy (see above) as compared with the multicopy ant. We, therefore, compared the properties of the antipporter activity in membrane vesicles derived from NM81, and TA15/pGM12 (Table II and Fig. 4). It is evident that both types of membrane vesicles exhibit not very different apparent \( K_a \) for Na+ (0.25 and 0.68 mM, respectively) with NM81 having slightly better apparent affinity. Since the acridine orange technique yields only a relative and a qualitative estimate of the antipporter activity, we measured directly initial rates of \( ^{22}\text{Na} \) transport as described (19). Identical results for the \( K_a \) values were obtained (not shown).

However, whereas NM81 vesicles exhibit an apparent \( K_a \) for Li+ of 0.86 mM, TA15/pGM12 vesicles show more than a 10-fold lower apparent \( K_a \) for the ion (60 \( \mu \)M). These results suggest that the ant system has a much higher apparent affinity to Li+ compared with the other system.

Working with right side out membrane vesicles derived from another wild type strain of *E. coli* (ML308225) Leblanc and his co-workers (10, 35) previously showed that the Na+/H+ antipporter activity is regulated by pH at the cytoplasmic side of the membrane. Since the cytoplasmic side of everted membrane vesicles is exposed to the medium pH we compared the effect of medium pH on the Na+/H+ antipporter activity of everted membrane vesicles derived from NM81 and TA15/pGM12 (Fig. 6).

The Na+/H+ antipporter activity of TA15/pGM12 show marked dependence on pH very similar in pattern to that previously observed for a wild type strain (35). Thus, at a low pH of 6.5, the activity is low, increasing with pH about 4-fold at pH 8.5. On the other hand, in marked contrast to this behavior the activity in NM81 vesicles is not affected at all over pH range of 6.5 to 8.5

Taken together the ant-dependent antipporter system differs from the additional system revealed in NM81 both in ion specificity and pH sensitivity.

### DISCUSSION

In this work we describe the disruption of the ant gene in the *E. coli* chromosome and its effect on growth tolerance to Na+, Li+, and pH and on the Na+/H+ antipporter activity in everted membrane vesicles.

We found that ant is not an essential gene. Growth of NM81, the \( \Delta \)ant strain, is normal both in minimal salt medium and in LB medium. Increasing the Na+ concentration uncovers an impaired adaptation in the \( \Delta \)ant strain. Thus, unlike \( \Delta \)ant+ strain, \( \Delta \)ant does not grow in LBK, pH 6.8, medium, used for the strain isolation, when 0.7 M NaCl is added. This Na+ sensitivity is markedly dependent on pH, increasing upon alkalization. At pH 7.5 in LBK medium, 0.5 M NaCl and at pH 8.6 in MTC medium, 0.1 M NaCl are sufficient to inhibit growth of \( \Delta \)ant. That this sensitivity is specific to Na+ ions is demonstrated by the fact that growth of \( \Delta \)ant strains is normal when KCl is added instead of NaCl at all pH values. Sensitivity to the effects of Li+ is also increased in the \( \Delta \)ant strain as compared with \( \Delta \)ant+. Whereas 100 mM LiCl inhibit growth of wild type cells in minimal medium A, 10 mM LiCl are already deleterious to \( \Delta \)ant cells.

The phenotype of \( \Delta \)ant seems to be due solely to the deletion of ant since we have not disrupted other open reading frames in its vicinity. The results are independent of whether the disrupting gene, coding for kanamycin resistance, was inserted in a reading frame with or against the direction of ant. In addition, \( \Delta \)ant phenotype is not strain-specific since JC7623 \( \Delta \)ant exhibited Na+ and Li+ sensitivity similar to NM81. Finally, the defective phenotype can be complemented by transformation with a plasmid bearing the ant gene.

Growth of \( \Delta \)ant strains is supported not only by carbon sources such as glycerol but also by ones such as melibiose in a Na+-dependent manner. These findings suggested that even though the \( \Delta \)ant strains have an impaired capacity to adapt to high salinity and to challenge Li+ toxicity it can still generate a Na+ gradient large enough to allow for growth on a carbon source whose transport requires Na+ (18). Indeed, from studies in everted membrane vesicles prepared

<table>
<thead>
<tr>
<th>Protein</th>
<th>NM81</th>
<th>TA15/pGM12</th>
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<tbody>
<tr>
<td>Na+</td>
<td>0.25</td>
<td>0.68</td>
</tr>
<tr>
<td>Li+</td>
<td>0.86</td>
<td>0.06</td>
</tr>
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**Fig. 6.** Effect of medium pH on the Na+/H+ antipporter activity of everted membrane vesicles of NM81 and TA15/pGM12. Activity of Na+/H+ antipporter was estimated as described in Fig. 4 at various pH values. Tricine, 10 mM, titrated with choline hydroxide was used to obtain pH 7.0-8.5 while MES-choline was used for pH 6.5. In addition to the buffers, the reaction mixture contained 140 mM KCl and 5 mM MgCl2. The activity of the Na+/H+ antipporter at each pH was estimated as described in Table II. Percent of dequenching due to addition of NaCl (8 mM) is depicted versus medium pH.
from the Δant strains a residual significant antiporter activity other than ant became evident. Because of this multiplicity of antiporter systems we shall call ant, antA. The residual activity detected in Δant strains will be tentatively called antB even though it is not yet established to be a product of a single gene.

The antB protein(s) displays kinetic properties different from those of antA, namely its activity in everted vesicles is practically independent of extravesicular pH while that of antA product is enhanced with increasing pH (10, 35). Since the measurements were conducted in everted membrane vesicles it is implied that antB is insensitive to intracellular pH while antA is very sensitive to this pH (10, 35). In addition, the apparent affinity for Li+ of antB at the cytoplasmic face of the membrane is about 15 times lower than that of antA.

The lack of antA and the properties of antB explain the phenotype of the Δant strain. The poor adaptation of the ΔantA strains to high Na+ is most likely due to lack of adequate Vmax, which in the wild type, is provided by combined activity of both antA and antB. The enhanced Li+ toxicity in the ΔantA strain can be explained in a similar manner. In the lower affinity of the antB system for Li+ ions is in line with the impaired capacity of ΔantA strains to challenge Li+ toxicity.

The increased sensitivity to Na+ with alkalization suggests the possibility that antA is mainly active at alkaline intracellular pH. This is corroborated by the finding that the antA antiporter activity in membrane vesicles is drastically dependent on intracellular pH, increasing about 4-fold upon alkalization. It is suggested that a Na+ leak into the cell, whether carrier mediated or not, increases with increasing pH. Therefore, at a given Na+ concentration increasing the pH increases the Na+ load, which in the absence of antA floods the antB system and brings about a collapse of cell homeostasis.

Growth of Δant strains at alkaline pH is normal provided Na+ ion concentration is kept low. These results suggest that, under these conditions, systems other than antA are sufficient to carry on homeostasis of cellular pH. Further studies are required to dissect in detail the mechanisms for pH regulation.

Multiplicity of transport systems for one substrate is not uncommon in bacteria. Thus, for example, this is the case for ions such as K+ (36) and Pi (37) and for several amino acids (38). The experimental demonstration of multiple Na+/H+ antiporters in E. coli is a novel finding. These data are in line with suggestions based on theoretical grounds (39) and raise many questions as to the nature of the alternative system(s), their interaction at the activity level, and their regulation at the gene level.

REFERENCES