Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2615–2619, May 1987
Biochemistry

Characterization of a Na^+/H^+ antiporter gene of *Escherichia coli*

(transport proteins/sequence)

EDWARD B. GOLDBERG*, TAMAR ARBEL*†, JIE CHEN*, RACHEL KARPEL‡, GEORGE A. MACKIE§, SHIMON SCHULDINER¶||, AND ETANA PADAN‡

*Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111; ‡Department of Molecular and Microbial Ecology, Life Science Institute, and ¶Department of Molecular Biology, Hadassah Medical School, Hebrew University, Jerusalem, Israel; and §Department of Biochemistry, Faculties of Medicine and Dentistry, University of Western Ontario, London, Canada N6A 5C1

Communicated by Herbert Weissbach, December 15, 1986

ABSTRACT **A mutant of *Escherichia coli* with increased Na^+/H^+ antiport activity was isolated and found by other workers to harbor two mutations [Niiya, S., Yamasaki, K., Wilson, T. H. & Tsuchiya, T. (1982) *J. Biol. Chem.* 257, 8902–8906]. The mutation that leads to increased Na^+/H^+ antiport (*ant*up) has now been separated and mapped. *ant*up maps in the vicinity of 0.5 min on the *E. coli* map, and the presence of this mutation alone in an isogenic pair raises antiport activity (V_{max}) by ≈4-fold. We also characterized cells bearing plasmids containing fragments of a 15-kilobase-pair segment of DNA between *carA* and *dnaJ*. A wild-type gene located within 2 kilobase pairs counterclockwise of *rpsT* increases the Na^+/H^+ antiport activity when present in multiple copies.**

In *Escherichia coli*, as in certain other bacteria, the Na^+/H^+ antiporter is the only known system for Na^+ extrusion (1–3). In *E. coli*, it maintains a Na^+ gradient ($Na^+_{in} < Na^+_{out}$) over a wide range of external Na^+ concentrations (4). The antiporter exchanges H^+ for Na^+ in a stoichiometry that is as yet undetermined but is most likely electrogenic (i.e., H^+/Na^+ is probably >1) (5–7). Thus, in growing cells, Na^+ efflux is driven by the electrochemical proton gradient generated by the primary proton pumps (1–7).

The resulting Na^+ gradient (directed inward) drives transport systems that catalyze Na^+/substrate symport (3). In *E. coli*, three such symporters are known—melibiose (8), glutamate (9), and proline (10). Na^+ symporters are common in halophiles and alkalophiles, and in the latter, the flagellar motor is driven by a Na^+ gradient as well (3). Since in many bacteria the Na^+ gradient is in steady-state equilibrium with the proton gradient, it may well serve as an energy buffer for the cell (5, 11). Thus, it is apparent that the Na^+/H^+ antiporter may play an important role in the bioenergetics of the prokaryotic cell.

In addition to its bioenergetic function, it has been suggested that the antiporter plays a role in pH homeostasis of bacterial cells in an alkaline environment (3, 5, 12, 13). In the alkalophilic bacterium *Bacillus alcalophilus*, it has been shown that a mutant lacking Na^+/H^+ antiporter activity lost its capacity to regulate its intracellular pH (pH_i) and to grow at alkaline pH (3). Another alkalophilic species that has an antiporter with high K_m for Na^+ requires Na^+ for growth and pH_i homeostasis. In the former strain, a Na^+ requirement for growth can only be demonstrated by rigorous exclusion of Na^+ (14). It was suggested that the rate of the antiporter activity, which has a low K_m for Na^+, is sufficiently high at the Na^+ concentrations contaminating the medium (3, 14).

An *E. coli* mutant that cannot regulate pH_i and therefore does not grow at alkaline pH_o has been isolated (15). Partial inhibition of the capacity to extrude Na^+ has been demonstrated in intact cells but not in isolated membrane vesicles of the mutant (15, 16). Since the mutation has been mapped in the α subunit of RNA polymerase, its relationship to Na^+/H^+ antiport activity and to regulation of pH_i is not yet clear (17).

Another *E. coli* mutant capable of growing on melibiose at levels of Li^+ toxic to the wild type was isolated and characterized by Niiya *et al.* (18). They mapped one mutation in this mutant in *melB* and presented evidence for a second mutation affecting Na^+/H^+ antiport activity (18, 19). Our interest in the Na^+/H^+ antiporter and its role in pH homeostasis of *E. coli* led us to study the second mutation, which we have designated *ant*up. As shown here, the *ant*up locus maps near *carA* (at ≈0.5 min on the *E. coli* map). Furthermore, transformation of appropriate cells with a plasmid carrying a wild-type chromosomal segment of the region substantially increases the Na^+/H^+ antiport activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains. For construction of the conjugal donor and recipient, we obtained *E. coli* K-12 strain W3133-2 (20) and RA11/Tn*10* from T. H. Wilson, W3133-2S from T. Tsuchiya, RV/F'MS1054 (21) from M. Malamy, and M5 (Beckwith collection) from J. Lopilato. All W3133 derivatives (including RA11) are prototrophic, streptomycin resistant (Sm^R), and Δ*lacZY*. RV is prototrophic, antibiotic sensitive, and Δ*lacIZYA*. MS1054 is *lacZ*$^+$Δ*Y*. M5 is leu::Tn*5*. In RA11/Tn*10*, tetracycline resistance is 85–90% linked to *mel* (T. H. Wilson, personal communication; unpublished experiments).

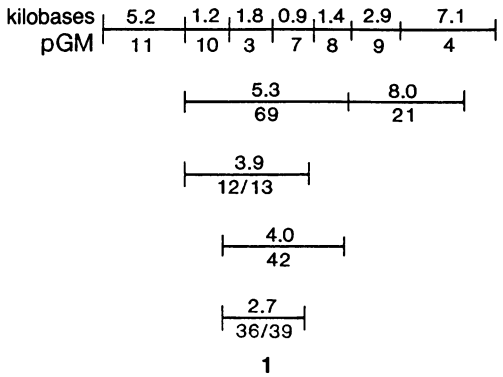
Phage Strains. P1 *vir* was obtained from J. Lopilato. P1.Leu::Tn*10* is a P1 lysate of *E. coli* M5.

Plasmids. All the pBR322 plasmids with their respective inserts have been described (22, 23) except for pGM42, -36, -39, and -69. Structure **1** illustrates the DNA segments included in each plasmid used. Nucleotide sequences in pGM7 and pGM8 are given in ref. 24.

Media. Cells were grown on L broth or in AK medium, which is minimal medium A (25) without sodium citrate supplemented with 0.6% glucose or 0.5% glycerol or melibiose, as required. Where indicated, sterile Li^+ or Na^+ was also added to yield 100 mM, unless stated otherwise. For plates, 1.5% Difco agar was added. LK broth uses KCl instead of NaCl. For selections and screenings, we used kanamycin or streptomycin (50 μg/ml) tetracycline (15 μg/ml), nalidixic acid (40 μg/ml), uracil (40 μg/ml), amino acids (40–50 μg/ml) (leucine, threonine, and arginine).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†Present address: Department of Genetics, Life Science Institute, Hebrew University, Jerusalem, Israel.
||Present address: Roche Institute of Molecular Biology, Nutley, NJ 07110.



Transduction. Cells were transduced with P1 *vir* phage essentially as described (26). After incubation, colonies were picked and streaked with toothpicks on additional screening plates.

Conjugation. For matings, a 20:1 ratio of midlogarithmic phase recipient to donor cells was mixed essentially as described (26). At intervals, 0.1 ml of mixture was diluted into 10 ml of AK buffer containing 50 μg of nalidixic acid per ml, mixed, and stored on ice. As controls, donor and recipient cultures (0.1 ml each) were treated similarly. An aliquot (0.1 ml) of each diluted sample was mixed with 3 ml of AK soft agar containing 0.8 mM 5-bromo-4-chloro-3-indolyl α-D-galactoside and 0.33 mM isopropyl α-D-thiogalactopyranoside and poured onto selection plates. The plates were incubated at 37°C for 36–48 hr.

Everted Membrane Vesicles. Everted membrane vesicles were prepared essentially as described by Rosen and coworkers (27, 28). Cells were grown in AK medium with 0.5% glycerol to logarithmic phase (200 Klett units; filter 42), washed, and concentrated ≈200-fold (4 ml/g wet weight) in TSCD (10 mM Tris·HCl, pH 7.5/140 mM choline chloride/0.25 M sucrose/0.5 mM dithiothreitol) at 4°C. DNase was added to 5 μg/ml and cells were lysed twice in a French pressure cell at 4000 psi (1 psi = 6.89 kPa). Unbroken cells were sedimented for 10 min at 12,000 × *g*. The supernatant was sedimented for 1 hr at 100,000 × *g* to pellet the everted membrane vesicles, which were washed once and resuspended in TSCD buffer at ≈10 mg of protein per ml and stored at −70°C.

Measurement of ΔpH. Acridine fluorescence was monitored to estimate ΔpH (6, 27, 29). The reaction mixture contained, in 2.5 ml, the following: 60–70 μg of membrane protein, 10 mM Tris-Hepes (pH 8), 140 mM choline chloride, 5 mM $MgCl_2$, 1 μM acridine orange, and 2 mM K^+ lactate. Fluorescence of acridine orange was monitored in an Eppendorf fluorimeter (model 1101M) and recorded by a Goerz recorder (model Re541, Vienna). Exciting light was provided by a mercury lamp and filtered through a 405- to 436-mm filter. Emission was measured through a 470- to 3000-nm filter.

Transport of ^{22}Na. The driving force for ^{22}Na uptake was a ΔpH (interior acid) generated by an ammonium ion gradient according to ref. 29. Everted membrane vesicles were loaded with NH_4Cl by incubating 600–700 μg of membrane protein in 10 ml of solution containing 150 mM NH_4Cl and 10 mM Tris-Hepes (pH 8.8) for 2.5 hr at room temperature. After centrifugation for 1.5 hr at 100,000 × *g*, the membranes were resuspended to 6 mg of membrane protein per ml in the loading solution. The loaded membranes were diluted 1:100 into a reaction mixture of 200 μl kept on ice (to reduce the flux rate) and containing 10 mM Tris-Hepes (pH 8.8), 150 mM choline chloride, 2.5 mM $MgSO_4$, and 35 μM ^{22}Na (specific activity, 20,000 cpm/nmol). At different time intervals, the samples were diluted into an ice-cold 2-ml reaction mixture lacking NaCl, filtered through Millipore filter membranes (average pore size, 0.22 μm; diameter, 25 mm) and washed with 2 ml of the latter solution. Radioactivity retained on the filters was determined on a Kontron γ counting system.

Expression of *ant*$^+$ Gene in Minicells. The minicell producing strain DR103 (30) was transformed with pGM12 or pAL199 (30). The latter is a pBR322 derivative, which carries a mutation in the *tet* gene. Therefore, it was used as a control for pGM12, which carries the *ant*$^+$ insert in the *tet* gene.

Isolation of minicells and labeling of their plasmid-encoded proteins were as described (30).

Protein concentration in cells and everted membrane vesicles was determined as described (31).

RESULTS AND DISCUSSION

The *ant*up Mutation Confers Li^+ Resistance. Li^+ inhibits melibiose transport in *E. coli* (32). Niiya *et al.* (18) found that a mutant (W3113-2S) selected for growth on melibiose in the presence of 10 mM Li^+ has two mutations. One mutation renders melibiose transport totally dependent on Na^+ or Li^+, whereas the wild type, W3113-2, is dependent on Na^+ or H^+ (the mutant allele is designated *melBLid*). The second mutation (*ant*up) augments Na^+/H^+ antiporter activity. It was suggested that this mutation was required for rapid excretion of the toxic Li^+, which enters the cell via the mutated melibiose transporter, thereby reducing the steady-state level of intracellular Li^+ (18).

To verify that it is the *ant*up mutation that confers Li^+ resistance on W3113-2S, we constructed the strain TA15 that has the *melBLid* mutation but not the *ant*up mutation. For this construction, we first made the strain W3113-2S/Tn*10* carrying Tn*10* 90% linked to the *melBLid* locus (see Fig. 1, first P1 cross). Only ≈10% of the tetracycline-resistant (*Tet*^{R}) transductants from the first cross, P1.RA11/Tn*10* × W3133-2S, were able to grow on melibiose in the presence of 100 mM LiCl. One of these was designated W3133-2S/Tn*10*. The Tn*10* from this strain was transduced by P1 into W3133-2, the wild-type *melB*$^+$ *ant*$^+$ strain, by selection for resistance to tetracycline (see Fig. 1, second P1 cross). In 90% of the *Tet*^{R} transductants, the *melBLid* marker was cotransduced. One of these, TA15, was isolated for further work. When grown on melibiose and 10 mM LiCl, colonies of TA15 were somewhat inhibited as compared to the double mutant W3133-2S, which was totally unaffected. Increasing the Li^+ concentration to 100 mM had a minor effect on the growth of the latter while drastically inhibiting the growth of TA15. Growth at 40°C increased this difference still further.

Increased Na^+/H^+ Antiporter Activity in W3133 Is Due to the *ant*up Mutation. By using acridine orange to monitor ΔpH at various Na^+ concentrations, Niiya *et al.* (18) demonstrated that everted membrane vesicles of *E. coli* W3133-2S (*ant*up) exhibit enhanced Na^+/H^+ antiport activity relative to *E. coli* W3133-2 (*ant*$^+$). To prove that the sensitivity of TA15 to high concentrations of Li^+ is due to the lack of augmented Na^+/H^+ antiport activity, antiport activity was assayed in everted membrane vesicles isolated from TA15. As shown in Fig. 2, strain TA15 has the same Na^+/H^+ antiport activity as strain W3133-2 (trace A) with *ant*$^+$ but much less than W3133-2S (trace B), which contains the *ant*up mutation. Therefore, we conclude that the *ant*up mutation enhances Na^+/H^+ antiport activity and enables the *melBLid* strain to grow on melibiose in the presence of Li^+. Based on the acridine orange technique, Niiya *et al.* (18) also suggested that the K_m of the Na^+/H^+ antiporter in everted vesicles isolated from *ant*up cells is lower than that in *ant*$^+$ vesicles. Since the acridine orange technique is indirect and semiquantitative, the finding was verified by measuring ^{22}Na transport directly in everted membrane vesicles. Rates of Na^+ transport at room temperature at Na^+ concentrations >40 μM were too fast to measure (our results and ref. 29), and

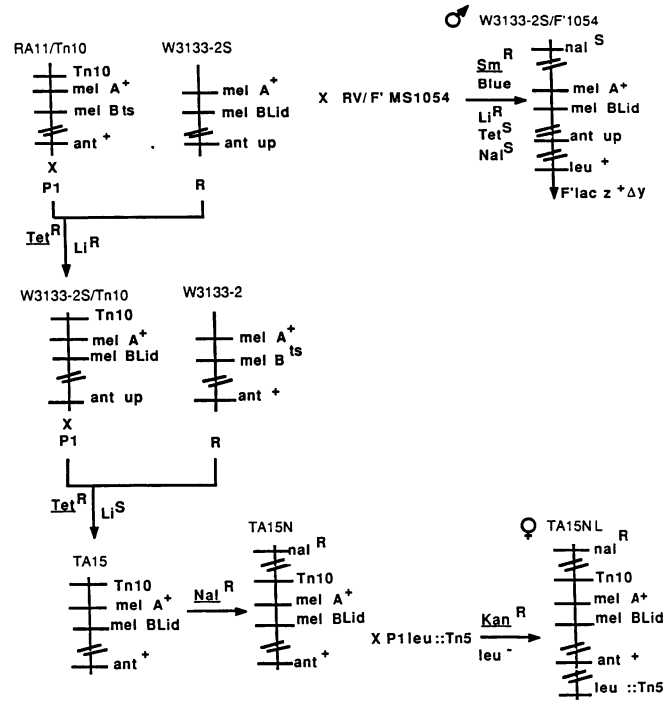


FIG. 1. Construction of bacterial strains. Relevant markers are given for each strain in their genetic order. (The one exception is Tn*10*. We do not know if Tn*10* should be above *melA* or below *melB*, but it is 90% linked to the *mel* operon.) Two slanted lines imply a looser genetic linkage than implied by the physical proximity in the figure. For an example of a transduction, the top left strain is infected (X) with P1, and the lysate is used to transduce the recipient (R) to its right. As an example of an F-duction, W3133-2S is crossed (X) with RV/F'MS1054. Selection procedures are underlined (e.g., *Tet*^{R}). Screening procedures are listed above the arrows (e.g., Li^R). Blue designates colonies blue on 5-bromo-4-chloro-3-indolyl γ-D-galactoside plates.

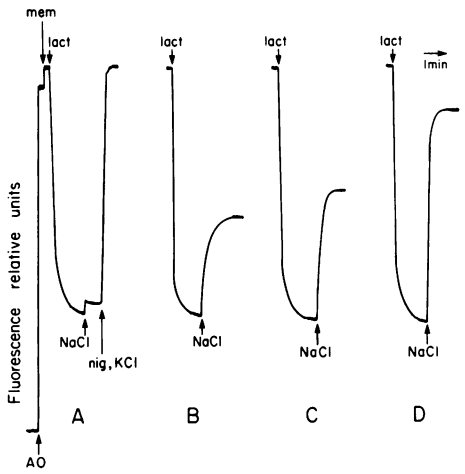


FIG. 2. The *ant* gene affects the Na^+/H^+ antiporter activity of everted membrane vesicles. Everted membrane vesicles were prepared (27, 28) from different strains and were used in experiments as follows: trace A, W3133-2, TA15, or TA15/pGM3; trace B, W3133-2S; trace C, TA15/pGM42; trace D, TA15/pGM12. Acridine orange was used to monitor ΔpH (27, 28). The reaction mixture contained (in 2.5 ml) 10 mM Tris-Hepes (pH 8), 140 mM choline chloride, and 5 mM $MgCl_2$. At the onset of the experiment, acridine orange (AO) was added to a 1 μM concentration, thereafter and as indicated, membrane vesicles (mem; 60–70 μg of membrane protein) and potassium D-lactate (lact; 2 mM) were added. The detailed protocol is shown in trace A. Where indicated, NaCl (10 mM), nigericin (1 μM), or KCl (10 mM) was added.

therefore we conducted the experiments on ice. Fig. 3 shows initial rates of ^{22}Na uptake for W3133-2S, and depicts a Lineweaver–Burk plot (*Inset*) for everted vesicles of TA15 (*ant*$^+$) and W3133-2S (*ant*up). From these data, it is evident that although V_{max} in W3133-2S is greater than that in TA15 (114 nmol per mg of protein per min and 30 nmol per mg of protein per min, respectively) [in addition, *cf.* Niiya *et al.* (18)], the K_m values are not significantly different (0.54 and 0.4×10^{-3} M, respectively). The apparent discrepancy with the K_m values given by Niiya *et al.* (18) may be due to the different techniques and conditions used in the measurements.

The *ant*up Mutation Is Located at ≈0.5 Min on the *E. coli* Map. To map the *ant*up mutation by conjugation, we converted TA15 to *nal*^{r} and *leu*$^-$ (Fig. 1, bottom row). This strain, TA15NL, was used as a recipient. The donor was W3133-2SF' *lacZ*$^+$Δ*Y*, made as illustrated in the top right cross of Fig. 1. The results of the cross are plotted in Fig. 4. Since *Tet*^{R} is 90% linked to *mel*, we used the loss of the *tet* marker as a measure of *mel* penetration. The entry of *ant*up between *leu*$^+$ and *tet*^{R} places it in the 8.4-min interval between 93.4 min and 1.8 min on the *E. coli* map (33). The observed interval between *leu* and *tet* in this experiment is 11.2 min and 2.2 min between *leu* and *ant*up. By normalizing for the discrepancy between the published and experimental distances, we estimate that the map distance between *leu* and *ant*up is estimated to be 1.65 min [i.e., (2.2)(8.4)/(11.2) = 1.65 min]. Therefore, the *ant* locus should be found in the vicinity of 0 min.

Transduction of a *carA*$^-$ *ant*up strain with P1 *carA*$^+$*ant*$^+$ shows 33% linkage between *carA* and *ant*up (data not shown). Thus, *ant*up lies between 0 and 1 min on the *E. coli* map.

Expression of *ant* Gene on Plasmids. With the localization of *ant*up in the vicinity of *carA*, we surveyed a series of plasmids covering ≈15 kilobase pairs counterclockwise from *car*,

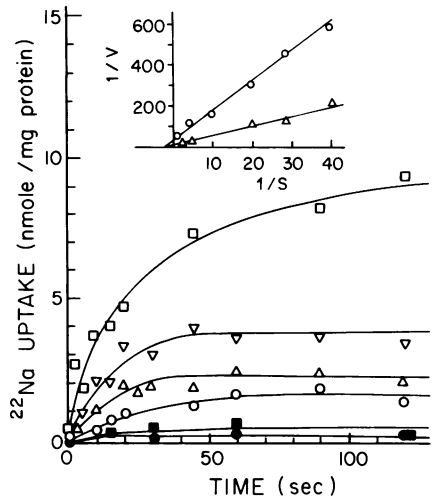


FIG. 3. The ΔpH-driven transport of ^{22}Na in everted membrane vesicles from strains bearing different *ant* genotypes. Everted membrane vesicles were prepared from strains of different *ant* genotypes as described in Fig. 1 and were loaded with NH_4Cl. The loaded membranes were diluted 1:100 into a reaction mixture of 300 μl kept at 4°C and containing (open symbols) 10 mM Tris-Hepes (pH 8.8), 150 mM choline chloride, 2.5 mM $MgSO_4$, and 35 μM $^{22}NaCl$ (specific activity, 20,000 cpm/nmol), or (solid symbols) a similar reaction mixture except that 150 mM NH_4Cl replaced choline chloride. The membrane suspensions were incubated for different times at 4°C, and ^{22}Na uptake was determined. ○, TA15/pGM3; △, W3133-2S; ▽, TA15/pGM42; □, TA15/pGM12. (*Inset*) Lineweaver–Burk plot of initial rate data to determine K_m and V_{max} for TA15 and W3133-2S. The dimensions for the ordinate ($1/v$) are min/μM and of the ordinate (1/s) are mM^{-1}. ○, TA15; △, W3133-2S.

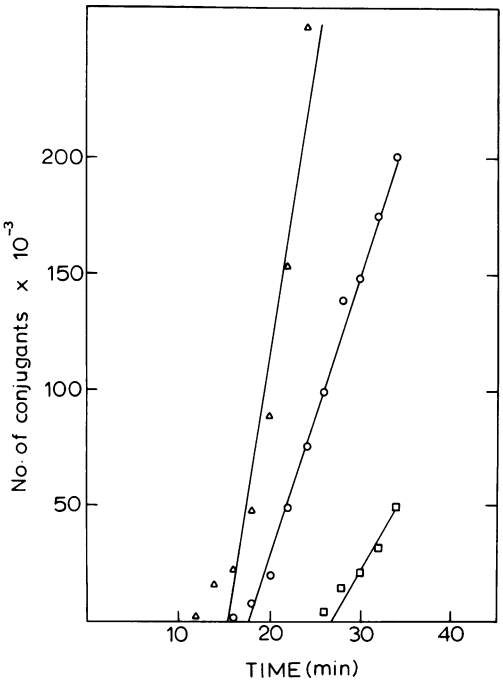


FIG. 4. Time of entry of *ant*up by interrupted mating. At intervals, cells were plated on AK agar containing melibiose and nalidixic acid (△) or melibiose, nalidixic acid, and 100 mM Li^+ (○). Colonies from the latter were streaked onto L-agar with tetracycline (□). The curves show the number of bacteria per ml in the conjugation mixture that can grow on nalidixic acid without leucine (△) and on melibiose, nalidixic acid, and 100 mM Li (○). The third curve (□) shows the number of bacteria per ml that have lost tetracycline resistance by recombination with the tetracycline-sensitive *mel* region on the entering donor chromosome. This was calculated from the fraction of colonies that did not grow when streaked on L broth with tetracycline.

extending almost to *dnaJ* (22, 23). The assumption was that although a fragment might have an ant^+ genotype, expression of multiple copies might yield the *ant*up phenotype in TA15 when grown on melibiose in the presence of Li^+. Fragments of this region were cloned into a pBR322 vector that retained the β-lactamase gene for selection with ampicillin. We transformed the vector into TA15 and looked for growth of colonies on L plates with ampicillin. Positive colonies were streaked on AK plates with melibiose and 100 mM LiCl (Table 1). Good growth (as exemplified by the control, W3133-2S) is scored as plus and poor growth (e.g., TA15) as minus.

Fig. 2 and Table 1 (column 3) show Na^+/H^+ antiport activity as determined with acridine orange in everted vesicles prepared from cells harboring plasmids with various inserts. Antiporter activity in the wild type is characterized by the non-plasmid-containing W3133-2 and TA15 strains, as well as the TA15/pBR322 (Fig. 2, trace A). The results show that all the plasmids containing inserts, which include at least the pGM3 and pGM7 regions in tandem (i.e., pGM36, -39, -42, -12, -13, and -69), exhibit a high level of antiport activity (Fig. 2, traces C and D; Table 1, column 3).

We verified these results by studying $^{22}Na^+$ uptake. Fig. 3 and Table 1 (column 4) show that antiport activity in some plasmid-containing cells can increase by up to 10-fold relative to wild-type activity (e.g., pGM12, -13, -39, and -42). In these strains, K_m or V_{max} cannot be measured accurately, as the rates are not linear with time at higher Na^+ concentrations. Lower Na^+ concentrations approach the contamination values (≈10 μM in these media).

In Fig. 3, it is also clear that the steady-state levels of sodium accumulation vary with the initial rates of uptake.

Table 1. Growth of colonies with ampicillin

Strain	Growth on melibiose in the presence of 100 mM Li^+	Na^+/H^+ antiporter activity	
		Acridine orange*	$^{22}Na^†$
W3133-2	−	0.1	
W3133-2S	+	0.48	7.0
TA15	−	0.07	2.2
TA15/pBR322	−	0.08	ND
TA15/pGM3	−	0.06	2.7
TA15/pGM7	−	0.05	ND
TA15/pGM36	+	0.72	ND
TA15/pGM39	+	0.64	17.0
TA15/pGM42	+	0.50	10.5
TA15/pGM12	+	0.76	20.0
TA15/pGM13	+	0.75	21.0
TA15/pGM69	+	0.47	7.6
TA15/pGM10	−	0.05	ND
TA15/pGM8	−	0.18	3.1
TA15/pGM4	−	0.14	ND
TA15/pGM21	−	0.23	2.2
TA15/pGM11	−	0.17	ND

ND, not done.
*Data are expressed as (enhancement of fluorescence after addition of 10 mM NaCl)/(fluorescence quenched after addition of lactate).
†Initial rate of ^{22}Na uptake at 35 μM Na^+ (nmol per min per mg of protein).

Only TA15/pGM12 approaches the steady state expected at thermodynamic equilibrium with a driving force of 2 pH units and H^+/Na^+ stoichiometry of 1.2 (4) (i.e., a concentration ratio of $^{22}Na^+_{in}/^{22}Na^+_{out}$ of 250). For everted vesicles with diminished Na^+/H^+ antiport activity, perhaps the passive Na^+ leakage rate is relatively high and the theoretical steady-state level cannot be achieved.

To further demonstrate the specificity of the *ant* locus, vesicles were tested for evidence of increased K^+/H^+ antiport activity. Fig. 5 shows that the K^+/H^+ antiport activity in a cell (TA15/pGM12) with the greatly increased Na^+/H^+ antiport activity is slightly affected. Analogous experiments with $CaCl_2$ also show that Ca^{2+}/H^+ antiport activity is not increased in TA15/pGM12 cells (data not shown).

A 30-kDa Open Reading Frame and Its Expression in Minicells. It is concluded that when excess wild-type ant^+ gene product is made, the level of antiport activity can rise above the wild-type level. Again, all the plasmids containing at least the inserts of pGM3 and pGM7 region in tandem, but not alone, produce increased antiport activity. The nucleotide sequence in this region is available (24): an open reading

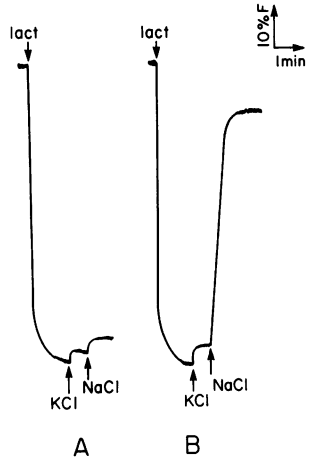


FIG. 5. An *ant* locus does not affect K^+/H^+ antiporter activity. Everted membrane vesicles were prepared from TA15 (trace A) or TA15/pGM12 (trace B) and ΔpH was measured from change in acridine orange fluorescence as described in Fig. 2. The reaction mixture contained 2 mM Tris D-lactate (lact) instead of K^+ lactate. Where indicated, KCl or NaCl was added at 10 mM.

frame capable of encoding a 30-kDa peptide starts at nucleotide 228 of pGM7 and spans further downstream into pGM8. The polypeptide is 262 amino acids long and has a calculated mass of 30 kDa. pGM12 (which includes the inserts of pGM3 and pGM7 in tandem) lacks the 30 carboxyl-terminal amino acids of the complete polypeptide. In addition, it contains stop codons in all three frames in the vector clockwise from the *Hin*dIII site. This will add the following three residues to the truncated carboxyl terminus: leucine, methionine, and arginine. Thus, antiport activity apparently does not require the 30 carboxyl-terminal amino acids. We have not been able to detect a promoter structure upstream of this open reading frame. This, together with the finding that pGM3 or part of it is also required for antiporter activity, suggests that either the promoter is located in pGM3 or another gene product encoded by pGM3 is required. The minicell producing strain DR103 (30) was transformed with pGM12 or pAL199, a pBR322 derivative bearing a mutated *tet* gene (30). When the minicells were pulse-labeled with [^{35}S]methionine and the proteins analyzed by NaDodSO_4/PAGE and autoradiography, two polypeptides with an apparent size of 28 and 33 kDa were detected when the *ant*$^+$ gene was present on the plasmid (pGM12) in addition to the β-lactamase, which was present in both minicell preparations (data not shown). The labeled polypeptides are recovered in the membrane fraction, while the β-lactamase remains in the soluble fraction.

CONCLUDING REMARKS

In this paper, we have localized a mutation, *ant*up, in the region of *carA*, that increases the Na^+/H^+ antiport activity of *E. coli*. In addition, we have cloned a wild-type gene located within 2 kilobase pairs counterclockwise of *rpsT*. When present in high copy number, *ant* increases the Na^+/H^+ antiport activity of *E. coli*. The insert encodes at least two polypeptides, one of which, a 30-kDa protein, has been sequenced (24). A truncated product of the latter devoid of 30 carboxyl-terminal amino acids (24 kDa) is fully active. Mapping data do not yet provide definitive evidence that the *ant*up mutation resides in this coding region. It is feasible to explain increased Na^+/H^+ antiport activity conferred by both the *ant*up mutation and the *ant*$^+$ gene by postulating regulatory functions. However, the simplest interpretation of these results is that one of the proteins is encoded by the structural *ant* gene for the Na^+/H^+ antiporter and that *ant*up is a mutation in this gene.

Note Added in Proof. Recent data show that a construct of pGM42 in which 417 nucleotides of the putative orf were deleted confers *ant*up phenotype. These data suggest that an insert that includes pGM3 and a segment of 600 nucleotides of the pGM7 insert suffices to encode the protein responsible for the *ant*up phenotype.

We would like to thank T. Tsuchiya for his gift of W3133-2S; T. H. Wilson, M. Malamy, and J. Lopilato for their generous help and advice; Hava Tamir for her technical help; and H. R. Kaback for helpful discussions. This work was supported by grants from Binational Science Foundation to S.S. and E.P. from National Institutes of Health Grant GM 13511 (to E.B.G.) and from the Medical Research Council of Canada (G.A.M.). E.B.G. was a John Simon Guggenheim Fellow during a sabbatical year at the Hebrew University in 1983–1984.

1. West, I. C. & Mitchell, P. (1974) *Biochem. J.* **144,** 87–90.
2. Harold, F. M. & Papineau, D. (1979) *J. Membr. Biol.* **8,** 45–62.
3. Krulwich, T. A. (1986) *J. Membr. Biol.* **89,** 113–125.
4. Castle, A. M., Macnab, R. M. & Shulman, R. G. (1986) *J. Biol. Chem.* **261,** 3288–3294.
5. Schuldiner, S. & Fishkes, H. (1978) *Biochemistry* **17,** 706–710.
6. Bassilana, M., Damiano, E. & Leblanc, G. (1984) *Biochemistry* **23,** 1015–1022.
7. Beck, J. C. & Rosen, B. P. (1979) *Arch. Biochem. Biophys.* **194,** 208–214.
8. Tsuchiya, T., Raven, J. & Wilson, R. (1977) *Biochem. Biophys. Res. Commun.* **76,** 26–31.
9. MacDonald, R., Lanyi, S. K. & Greene, R. V. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3167–3170.
10. Stewart, L. M. D. & Booth, I. R. (1983) *FEMS Microbiol. Lett.* **19,** 161–164.
11. Brown, I. I., Gralperin, M. Y., Glagolev, A. N. & Skulachev, V. P. (1983) *Eur. J. Biochem.* **134,** 345–349.
12. Padan, E., Zilberstein, D. & Rottenberg, H. (1976) *Eur. J. Biochem.* **63,** 533–541.
13. Padan, E., Zilberstein, D. & Schuldiner, S. (1981) *Biochim. Biophys. Acta* **650,** 151–166.
14. Krulwich, T. A., Guffanti, A. A., Fong, M. Y., Falk, L. & Hicks, D. B. (1986) *J. Bacteriol.* **165,** 884–889.
15. Zilberstein, D., Padan, E. & Schuldiner, A. (1980) *FEBS Lett.* **116,** 177–180.
16. Giffard, P. M., Rowlland, G. C., Kroll, R. G., Stewart, L. M. D., Bakker, E. P. & Booth, I. R. (1985) *J. Bacteriol.* **164,** 904–910.
17. Booth, I. R. (1985) *Microbiol. Rev.* **49,** 359–378.
18. Niiya, S., Yamasaki, K., Wilson, T. H. & Tsuchiya, T. (1982) *J. Biol. Chem.* **257,** 8902–8906.
19. Shiota, S., Yazyu, H. J. & Tsuchiya, T. (1984) *J. Bacteriol.* **160,** 445–447.
20. Lopilato, J., Tsuchiya, T. & Wilson, T. H. (1978) *J. Bacteriol.* **134,** 147–156.
21. Malamy, M. (1966) *Cold Spring Harbor Lab.* **31,** 189–201.
22. Mackie, G. A. (1980) *J. Biol. Chem.* **255,** 8928–8935.
23. Mackie, G. A. & Parsons, G. D. (1982) *Can. J. Biochem.* **60,** 338–346.
24. Mackie, G. A. (1986) *Nucleic Acids Res.* **14,** 6965–6981.
25. Davis, G. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60,** 17–28.
26. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
27. Ambudkar, S. V., Zlotnick, G. W. & Rosen, B. P. (1984) *J. Biol. Chem.* **259,** 6142–6146.
28. Rosen, B. P. (1986) *Methods Enzymol.* **125,** 328–336.
29. Nakamura, T., Hsu, C. M. & Rosen, B. P. (1986) *J. Biol. Chem.* **201,** 678–683.
30. Padan, E., Arbel, T., Rimon, A., Bar Shira, A. & Cohen, A. (1983) *J. Biol. Chem.* **258,** 5666–5673.
31. Bradford, W. (1976) *Anal. Biochem.* **72,** 248–254.
32. Tsuchiya, T., Lopilato, J. & Wilson, T. H. (1978) *J. Membr. Biol.* **42,** 45–59.
33. Bachmann, B. G. (1983) *Microbiol. Rev.* **47,** 180–230.