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Relationship between the Na^+/H^+ antiporter and Na^+/substrate symport in *Bacillus alcalophilus*

(nonalkalophilic mutant/efflux/vesicles)

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ABSTRACT **The Na^+/H^+ antiporter of the obligate alkalophile *Bacillus alcalophilus* facilitates growth at alkaline pH and precludes growth below pH 8.5. Thus, nonalkalophilic mutant strains do not exhibit Na^+/H^+ antiport activity and, interestingly, such strains concomitantly lose the ability to catalyze Na^+-dependent accumulation of α-aminoisobutyrate [Krulwich, T. A., Mandel, D. G. Bornstein, R. F. & Guffanti, A. A. (1979) *Biochem. Biophys. Res. Commun.* 91, 58–62]. Several other Na^+-dependent transport systems are now documented in vesicles from the wild-type strain, and it is demonstrated that these systems are defective in vesicles from the nonalkalophilic mutant KM23. Surprisingly, the defect seems to result not from the loss of Na^+/H^+ antiport activity *per se* but from a pleiotropic defect in the Na^+/substrate symporters themselves. Monensin, an ionophore that catalyzes Na^+/H^+ exchange, does not restore respiration-driven Na^+/substrate symport in KM23 vesicles. Moreover, with KM23 vesicles, efflux of α-aminoisobutyrate, L-malate, and L-aspartate down their respective concentration gradients is not stimulated by Na^+, in contrast to the observations with wild-type vesicles. Because monensin should ameliorate a simple defect in Na^+/H^+ antiport activity and the antiporter should not be required for Na^+/substrate symport down a concentration gradient, the results suggest that there may be a direct relationship between the antiporter and various Na^+/substrate symporters. One possibility is that the systems share a Na^+-translocating subunit.**

Although many bacterial transport systems catalyze the coupled movement of protons with substrate (i.e., H^+/substrate symport), a substantial number catalyze Na^+/substrate symport in analogy with eukaryotic transport systems (1–6). Unlike eukaryotes, however, most bacterial cells do not possess a primary Na^+ pump (e.g., Na^+, K^+-ATPase), and an overall mechanism proposed by Mitchell (7) that involves indirect coupling between various Na^+/substrate symporters and an antiporter catalyzing H^+/Na^+ exchange has received strong experimental support (2–13). By this means, Na^+/substrate symport is driven thermodynamically by the electrochemical gradient of protons, which functions to maintain a Na^+ gradient ($Na^+_{in} < Na^+_{out}$) through the activity of a Na^+/H^+ antiporter.

Because the Na^+/H^+ antiporter and the Na^+/substrate symporters are presumed to be distinct, substrate-specific catalysts, it is notable that nonalkalophilic mutants of *Bacillus alcalophilus* deficient in Na^+/H^+ antiport activity are also defective in Na^+/aminoisobutyrate (AIB) symport (13–15). Although diminished steady-state levels of Na^+-dependent AIB accumulation secondary to loss of antiport activity are expected, the initial rate of AIB transport is also severely altered. Furthermore, addition of monensin, an ionophore that catalyzes Na^+/H^+ exchange, does not restore AIB accumulation in the nonalkalophilic mutant (15). Importantly, each of several dozen independently isolated nonalkalophilic strains lacks both Na^+/H^+ antiport and Na^+/AIB symport activity (15); and at least a dozen revertants have regained both activities simultaneously, as well as the characteristic wild-type properties of the respiratory chain (15, 16). For these reasons, the possibility was considered that there may be a more direct relationship between the Na^+-translocating antiport and symport systems than generally thought to be the case. The experiments presented here support this notion by demonstrating that mutational loss of Na^+/H^+ antiport activity in *B. alcalophilus* KM23 simultaneously leads to a pleiotropic defect in Na^+-dependent substrate translocation.

MATERIALS AND METHODS

Growth of Cells and Preparation of Membrane Vesicles. *B. alcalophilus* (American Type Culture Collection 27647) and the nonalkalophilic derivative KM23 were grown on L-malate-containing media at pH 10.5 and 6.8, respectively (4, 15). Membrane vesicles were prepared by osmotic lysis of lysozyme-induced protoplasts (12, 17), suspended in 100 mM potassium carbonate buffer (pH 9.0) containing 10 mM $MgSO_4$, frozen in liquid nitrogen, and stored at −70°C.

Transport Assays. Uptake of radioactive substrates was measured by filtration as described (4). The assay mixtures contained 100 mM potassium carbonate (pH 9.0), 10 mM $MgSO_4$, and a given radioactive substrate at a specified concentration and specific activity. Where indicated, 10 mM sodium carbonate was also added. Potassium ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) at final concentrations of 20 mM and 2 mM, respectively, were used as an artificial electron donor system, and the reaction mixtures were gassed with water-saturated oxygen. Uptake was initiated by the addition of 20 μl of a membrane suspension containing 100 μg of membrane protein to 80 μl of reaction mixture. The reactions were terminated by rapid dilution with potassium carbonate buffer, immediate filtration through nitrocellulose filters (pore diameter 0.45 μm), and one wash with the same buffer (4). Radioactivity was determined by liquid scintillation spectrometry.

Carrier-mediated efflux down a concentration gradient was measured as described (18–20). Vesicles were concentrated to approximately 30 mg of protein per ml in 100 mM potassium carbonate (pH 9.0) containing 10 mM $MgSO_4$ and, where indicated, 10 mM sodium carbonate. A small sample of a given radioactive substrate was added to a specified concentration and specific activity, and the suspension was incubated for 4 hr at 5°C to allow equilibration with the intravesicular space. Efflux reactions were initiated by diluting 2 μl of the equilibrated

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Abbreviations: AIB, α-aminoisobutyrate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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vesicle suspension into 2 ml of a given buffer, followed by immediate dispersion with a Vortex mixer. After incubation for various times at 25°C, the vesicle suspensions were filtered through 0.45 μm nitrocellulose filters and the retained vesicles were washed with 2 ml of the reaction buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. The percentage of solute retained was calculated by comparison to zero-time points obtained as described (18–20). In some experiments, there was an initial, rapid loss of solute that is unrelated to carrier-mediated efflux (18–20). The half-time of efflux ($t_{1/2}$) was calculated from the first-order rate of efflux after an initial rapid loss of solute.

Protein Assay. Protein was determined by the method of Lowry *et al.* (21), using egg white lysozyme as standard.

Chemicals. L-[*U*-^{14}C]Malic acid (55 mCi/mmol) was obtained from Amersham/Searle, and L-[2,3-^{3}H]aspartic acid (25 Ci/mmol) and α-amino[1-^{14}C]isobutyric acid (53.2 mCi/mmol) were from New England Nuclear (1 Ci = 3.7×10^{10} becquerels). Monensin was generously provided by R. L. Hamill (Eli Lilly). All other materials were reagent grade of highest available purity.

RESULTS

As shown in Fig. 1, in the presence of ascorbate/TMPD and Na^+, membrane vesicles from wild-type *B. alcalophilus* take up AIB rapidly and achieve a steady-state level of accumulation in approximately 10 min (not shown), whereas in the absence of Na^+ both the initial rate of uptake and the steady-state level of accumulation are markedly diminished. With vesicles prepared from the nonalkalophilic strain KM23, on the other hand, there is relatively little transport of AIB in the presence or absence of Na^+ under the same conditions, and the defect in Na^+-dependent AIB accumulation is apparent from the onset of the transport reaction (i.e., as early as 5 sec). As found previously with intact cells (15), KM23 vesicles exhibit appreciable AIB uptake at pH 7.0, but uptake at this pH is not Na^+ dependent (data not shown). Thus, KM23 has an AIB porter that is functional at neutral pH, but it is no longer Na^+ dependent. It is also evident from the data in Fig. 1 that addition of monensin to KM23 vesicles has no effect whatsoever on AIB accumulation. This observation is important because it is expected from straightforward chemosmotic considerations that the ionophore would provide the system with the ability to exchange internal Na^+ for external H^+ and thus restore Na^+-dependent AIB accumulation to some extent at least in these vesicles. The lack of a restorative effect of monensin in this regard is not due to an inability of the ionophore to function at high pH (4). Also, previous work (4) indicates that monensin catalyzes Na^+/H^+ exchange even in the presence of high K^+ concentrations (i.e., under the conditions described in Fig. 1). As an added control, however, KM23 vesicles were prepared in 100 mM sodium carbonate buffer at pH 9.0, and monensin was added at concentrations ranging from 0.1 to 2.0 μg/ml. No AIB uptake was observed in the presence of ascorbate/TMPD at any of the monensin concentrations employed.

Because the protonmotive force is relatively low in alkalophilic bacteria (4, 14), it is reasonable to expect that such bacteria utilize the electrochemical Na^+ gradient for active transport, and several Na^+-dependent symport systems have been described in alkalophiles (4, 14, 22, 23). Although detailed ex-

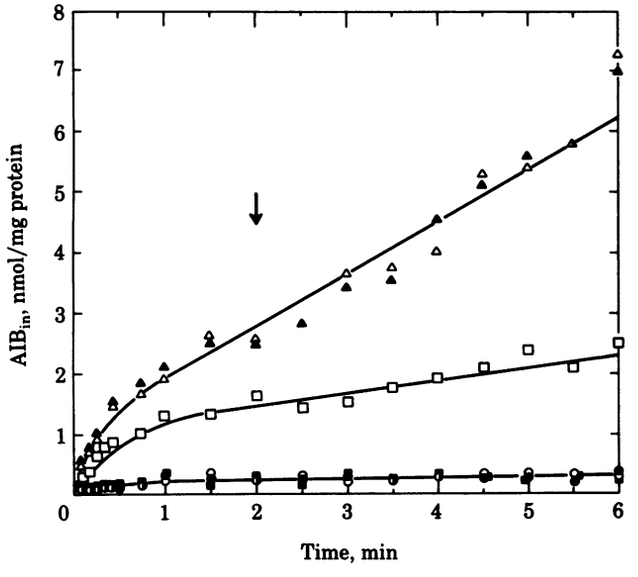

FIG. 1. Effect of Na^+ and monensin on AIB uptake by membrane vesicles from wild-type *B. alcalophilus* and from strain KM23. K^+-loaded vesicles from wild type (△, ▲, □) or KM23 (○, ●, ■) were suspended in 100 mM potassium carbonate (pH 9.0) containing 10 mM $MgSO_4$. One-third of the samples (■, □) contained no added Na^+, while the other two-thirds were supplemented with 1 mM sodium carbonate (pH 9.0) (●, ○, ▲, △). At 2 min, either monensin at 2 μg/ml in ethanol (▲, ●) or ethanol alone (△, ○) was added (arrow). All reaction mixtures contained 40 μM [1-^{14}C]AIB (26 mCi/mmol). Uptake was initiated by adding 20 mM potassium ascorbate and 2 mM TMPD, and the reactions were carried out at 30°C with aeration. At the times indicated, samples containing 100 μg of membrane protein were diluted, filtered, and washed once with the reaction buffer (4).

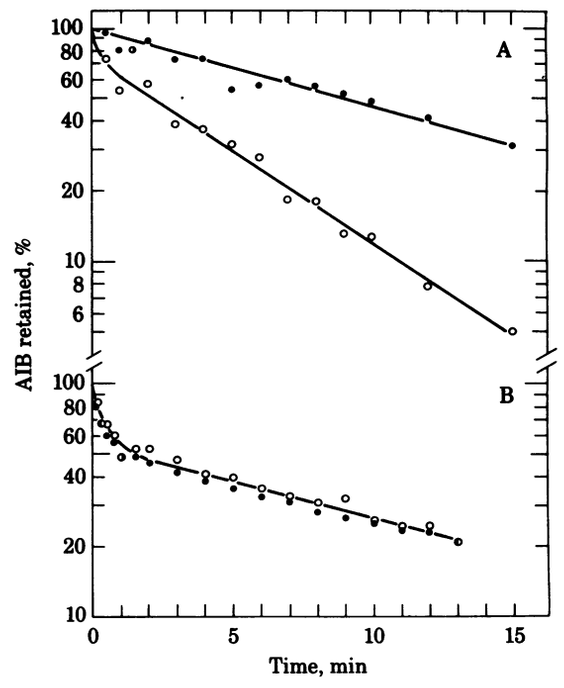

FIG. 2. Effect of Na^+ on AIB efflux. Membrane vesicles from wild-type *B. alcalophilus* (*A*) and KM23 (*B*) were washed and suspended to 30 mg of membrane protein per ml in 100 mM potassium carbonate (pH 9.0) containing 10 mM $MgSO_4$. A small sample of [1-^{14}C]AIB (4.9 mCi/mmol) was added to the membrane suspension to a final concentration of 10 mM. The suspension was divided into two parts, one of which was brought to 10 mM in Na^+ (○) and the other 10 mM in K^+ (●), in both cases with bicarbonate salts. After equilibration for 4 hr at 5°C, 2-μl aliquots were diluted 1:1000 into the same buffer in which they were suspended. At the times indicated, samples were filtered and washed immediately with 2 ml of buffer. The percentage of AIB retained was determined by comparison with zero-time points (24 and 19 nmol/mg of protein for *B. alcalophilus* and KM23 vesicles, respectively). Each point represents the average of at least two determinations.

periments will not be presented, studies similar to those shown in Fig. 1 demonstrated that the defect in Na^+-dependent AIB transport observed in KM23 vesicles can be extended to a number of other Na^+/substrate symport systems. Thus, in addition to AIB, wild-type vesicles actively transport L-leucine, L-serine, L-aspartate, and L-malate in the presence of ascorbate/TMPD, and the initial rates of uptake and steady-state levels of accumulation are markedly increased by Na^+. For example, initial rates of L-malate and L-aspartate transport are 9.3 and 0.9 nmol/min per mg of protein, respectively, in the presence of Na^+ and 3.6 and 0.2 nmol/min per mg of protein in the absence of the cation. Under the same conditions, KM23 vesicles exhibit little or no uptake of any of these solutes in either the presence or the absence of Na^+, and addition of monensin in the presence of Na^+ does not stimulate uptake. Parenthetically, moreover, preliminary experiments suggest that the transport of these solutes is mediated by distinct carrier systems, because L-aspartate does not significantly inhibit the uptake of L-malate or vice versa.

These experiments document a pleiotropic loss of Na^+-dependent active transport associated with a genetically induced lesion in the Na^+/H^+ antiporter, and they suggest that the defect results from a direct effect of the mutation on the symporters. In order to test this suggestion more rigorously, passive, carrier-mediated efflux was examined in the absence of imposed ion gradients. By this means, symport function can be studied under conditions in which the antiporter is not involved in the overall translocation process (20).

The effect of Na^+ on passive, carrier-mediated efflux of AIB, L-malate, and L-aspartate down their respective concentration gradients was investigated by examining rates of efflux in wild-type and KM23 vesicles in the presence and absence of added Na^+. Concentrated vesicle suspensions were equilibrated with a given substrate with and without Na^+ and then diluted 1:1000 into the same buffer without substrate. Clearly, as reported with the Na^+/melibiose symporter in *Escherichia coli* (20), in wild-type vesicles (tops of Figs. 2, 3, and 4), the rate of efflux of each substrate is enhanced by a factor of 3–4 in the presence of the cation ($t_{1/2}$ values for efflux of AIB, L-malate, and L-aspartate are 10.4, 18.4, and 12 min, respectively, in the absence of Na^+ and 3.2, 5.4, and 3.6 min, respectively, in the presence of Na^+). Strikingly, however, when precisely the same operations are performed with KM23 vesicles (bottoms of Figs. 2, 3, and 4), the rates of AIB, L-malate, and L-aspartate efflux are essentially identical in the presence and absence of Na^+ ($t_{1/2}$ values of 8.5, 8.8, and 9.3 min, respectively, are observed for each substrate with and without Na^+). In addition, it is important that monensin has no significant effect on the efflux rates observed in KM23 vesicles in the presence of Na^+ (data not presented).

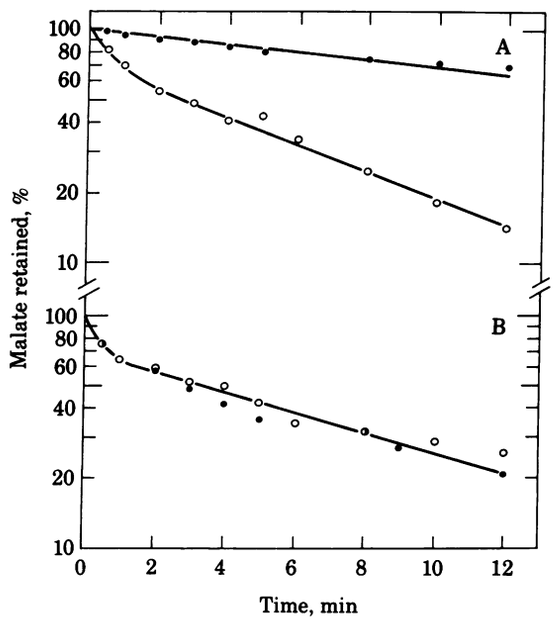

FIG. 3. Effect of Na^+ on malate efflux. Membrane vesicles from wild-type *B. alcalophilus* (*A*) and KM23 (*B*) were prepared at pH 9.0. The vesicles were loaded with 10 mM L-[*U*-^{14}C]malate (12.5 mCi/mmol) in the presence (○) or the absence (●) of Na^+ and assays were performed as in Fig. 1 and *Materials and Methods*. Zero-time values were 21 and 17 nmol/mg of protein for *B. alcalophilus* and KM23 vesicles, respectively.

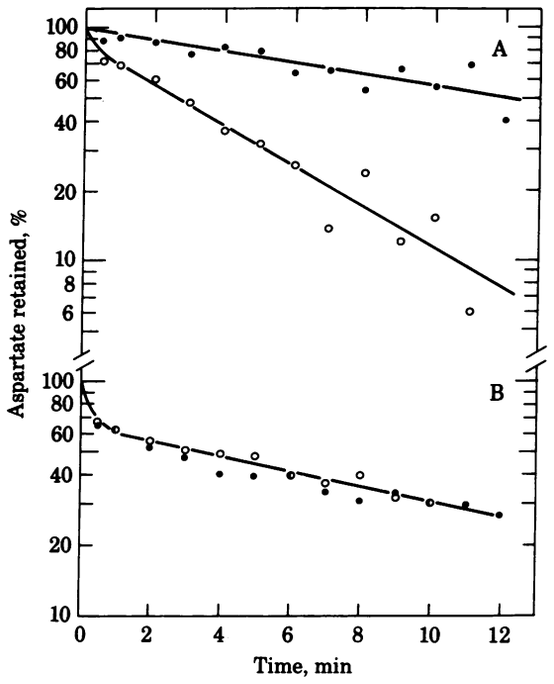

FIG. 4. Effect of Na^+ on aspartate efflux. Membrane vesicles from wild-type *B. alcalophilus* (*A*) and KM23 (*B*) were prepared at pH 9.0. The vesicles were loaded with 10 mM L-[2,3-^{3}H]aspartate (2.5 mCi/mmol) in the presence (○) or the absence (●) of Na^+ and assayed. Zero-time values were 22 and 17 nmol/mg of protein for *B. alcalophilus* and KM23 vesicles, respectively.

DISCUSSION

The preliminary results presented here support a unique and interesting conclusion; namely, that the interrelationship between the Na^+/H^+ antiporter and Na^+/substrate symport in *B. alcalophilus* is more immediate than generally assumed. Thus, the genetically induced defect in the Na^+/H^+ antiporter in KM23 is accompanied by a pleiotropic defect in at least three respiration-driven Na^+/substrate symport systems, and the defect is clearly apparent during the earliest phase of the transport reactions. Furthermore, the defect in Na^+/substrate symport in KM23 vesicles is not reversed by monensin, an ionophore with Na^+/H^+ antiport activity. Finally and most definitively, under conditions in which Na^+-dependent substrate translocation can be studied independent of Na^+/H^+ antiport activity (i.e., carrier-mediated efflux down a concentration gradient), KM23 vesicles exhibit a complete lack of Na^+/substrate symport.

Before an explanation is attempted, a few additional points are pertinent. The possibility that the wild type, but not KM23, requires Na^+ for generalized membrane integrity is ruled out

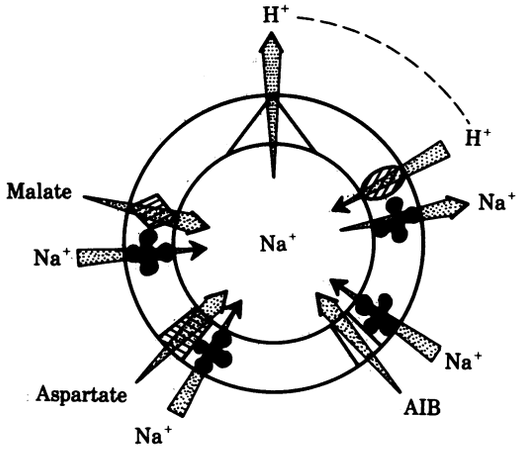

FIG. 5. Conceptual model describing the relationship between the Na^+/H^+ antiporter and various Na^+/substrate symporters in *B. alcalophilus*. The "cloverleaves" represent the Na^+-translocating subunit common to the Na^+/H^+ antiporter and each Na^+/substrate symporter. Other symbols indicate substrate-specific subunits unique to each translocation complex. Varying thickness of the arrows is meant to depict the direction of the electrochemical gradient of each substrate.

by previous studies demonstrating that vesicles prepared without Na^+ generate an electrochemical proton gradient (13), exhibit K^+/H^+ antiport activity (13), and synthesize ATP under appropriate conditions (§). It is also unlikely that the defect in Na^+-dependent substrate translocation in KM23 reflects an absolute lack of substrate-specific carriers. Thus, KM23 catalyzes AIB transport at pH 7.0 and grows well at this pH with L-malate as sole carbon source.

The findings can be rationalized by the speculative conceptual model presented in Fig. 5. As shown, the Na^+/H^+ antiporter and the individual Na^+/AIB, Na^+/malate, and Na^+/aspartate symporters are depicted as oligomers consisting of at least two dissimilar subunits. One subunit, common to each "translocation complex" and encoded presumably by the gene mutated in KM23, is responsible for Na^+ translocation. The other subunits, encoded by a series of unrelated genes, are unique to each complex and involved in the translocation of specific substrates. It is obvious from this scheme that a mutation in the gene coding for the "Na^+ subunit" would result in a pleiotropic defect in all Na^+-translocating systems, which include both the Na^+/H^+ antiporter and various Na^+/substrate symporters. On the other hand, mutations in genes coding for "substrate-specific subunits" would lead to unique defects in individual symporters.

As such, the model is reminiscent of the well-documented situation with the phospho*enol*pyruvate–protein phosphotransferase system (PTS; EC 2.7.3.9), in which genetic lesions in either of the general components (i.e., enzyme I or the histidine-containing protein) cause pleiotropic loss of PTS-mediated transport, and mutations in the sugar-specific components (enzyme IIs) are unique (24, 25). In addition, the model is somewhat analogous to the suggestion of Hong (26) that the H^+/substrate symport systems in *E. coli* share a H^+-translocating subunit. In any event, the presence of such a common Na^+ subunit might also be related to the interesting finding that induction of the Na^+/melibiose symporter in *E. coli* results in enhanced Na^+ efflux activity (27). Furthermore, Zilberstein *et al.* (28) have recently reported that mutation in a single gene locus in *E. coli* causes sensitivity to growth at alkaline pH, loss of Na^+ extrusion, and defective Na^+-dependent glutamate and melibiose transport. Although these properties may result from loss of Na^+/H^+ antiport activity *per se*, the defect in Na^+-dependent active transport may be a more direct result of the mutation, as suggested here for *B. alcalophilus* KM23.

Finally, it should be emphasized that, while KM23 is defective in Na^+/substrate symport, it does catalyze Na^+-independent transport at neutral pH, and preliminary evidence suggests that the mechanism involves H^+/substrate symport (15). Although it is not clear whether or not the loss of Na^+ coupling is accompanied by the appearance of H^+ coupling, the latter would be a feasible mechanism of energization in the pH range in which the mutant grows. Similarly, in *E. coli* it has been reported (29) that melibiose can be transported in symport with either Na^+ or H^+, but the mechanistic basis of the flexibility is not known.

Note Added in Proof. In addition to exhibiting pleiotropic defects in growth at alkaline pH and Na^+-dependent active transport of glutamate and melibiose, the *E. coli* Na^+/H^+ antiport mutant (28) is also defective in Na^+-dependent efflux of glutamate and melibiose (D. Zilberstein, S. Schuldiner, and E. Padan, personal communication).

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