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Sodium/Proton Antiporter in *Streptococcus faecalis*

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***Streptococcus faecalis*, like other bacteria, accumulates potassium ions and expels sodium ions. This paper is concerned with the pathway of sodium extrusion. Earlier studies (D. L. Heefner and F. M. Harold, Proc. Natl. Acad. Sci. USA 79:2798–2802, 1982) showed that sodium extrusion is effected by a primary, ATP-linked sodium pump. I report here that cells grown under conditions in which sodium ATPase is not induced can still expel sodium ions. This finding suggested the existence of an alternate pathway. Sodium extrusion by the alternate pathway requires the cells to generate a proton motive force. This conclusion rests on the following observations. (i) Sodium extrusion required glucose. (ii) Sodium extrusion was observed at neutral pH, which allows the cells to generate a proton motive force, but not at alkaline pH, which reduces the proton motive force to zero. (iii) Sodium extrusion was inhibited by the addition of dicyclohexylcarbodiimide and of proton-conducting ionophores. (iv) In response to an artificial pH gradient (with the exterior acid), energy-depleted cells exhibited a transient sodium extrusion which was unaffected by treatments that dissipated the membrane potential and which was blocked by proton conductors. I propose that streptococci have two independent systems for sodium extrusion: an inducible sodium ATPase and a constitutive sodium/proton antiporter.**

The significance of sodium circulation for the physiology of bacteria is well recognized (4, 17, 18, 21). Growing bacterial cells extrude sodium ions actively and maintain a sodium concentration gradient directed inward. Sodium extrusion by bacteria is generally attributed to secondary antiport of sodium for protons, which is energized by the proton motive force (3, 17, 19). The sodium gradient serves as a driving force for transport systems catalyzing sodium/substrate symport (18). In addition, sodium/proton antiporters are thought to be involved in the regulation of the cytoplasmic pH (17, 20).

From earlier studies of sodium extrusion by *Streptococcus faecalis*, Harold and his collaborators concluded that streptococci expel Na^+ by means of an ATP-driven primary pump that exchanges sodium ions for protons (8–10). Heefner and Harold also demonstrated sodium/proton antiport activity in both cells and membrane vesicles, but they regarded this activity as an artifact arising from proteolytic damage to the ATPase (7, 9). They suggested that the ATPase is composed of two subunits, i.e., a Na^+/H^+ antiporter and an associated catalytic subunit. Damage to this modular pump could alter the association of the subunits, resulting in the appearance of sodium/proton antiport activity (7, 9, 10).

Subsequent research on the sodium ATPase led me to question the view that *S. faecalis* has only a single system for sodium extrusion and that the Na^+/H^+ antiport activity arises by proteolytic damage to the sodium ATPase. In a study of potassium accumulation via the KtrII system of *S. faecalis*, Kakinuma and Harold found that the system responsible for potassium accumulation is the sodium ATPase (5, 12). That is, the sodium ATPase apparently exchanges Na^+ for K^+ rather than for H^+. It seemed unlikely, therefore, that proteolytic degradation of this enzyme would generate Na^+/H^+ antiport activity. Moreover, the sodium ATPase of *S. faecalis* is an inducible enzyme that is produced only when the cells are grown in medium rich in Na^+ (12, 13). Cells grown in medium lacking sodium ion would then be unable to expel sodium, which again seemed implausible.

In fact, cells grown under conditions that do not induce production of the sodium ATPase were still capable of energy-dependent sodium extrusion. Sodium extrusion under these conditions required the cells to generate a proton motive force, with the cytoplasm alkaline and negative. I propose that *S. faecalis* produces two systems for Na^+ extrusion: a secondary Na^+/H^+ antiporter, which appears to be constitutive, and an inducible primary sodium pump.

MATERIALS AND METHODS

Organisms and growth media. All the experiments were conducted with *S. faecalis* ATCC 9790, which was generously supplied by F. M. Harold (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.), or with mutant AS25 derived from this strain. AS25 was kindly supplied by H. Kobayashi (Chiba University, Chiba, Japan). Organisms were grown on the standard complex media, modified as follows (amounts are per liter). For NaTY, 2.5 g of Difco tryptone, 1.25 g of Difco yeast extract, 10 g of glucose, and 8.5 g of Na_2HPO_4 were added. For KTY, tryptone, yeast extract, and glucose as above plus 10 g K_2HPO_4 were added. For TrisTY, tryptone, yeast extract, and glucose as above plus 0.2 M Tris base and 0.05 M H_3PO_4 were added. The pH was brought to 7.5 with NaOH, KOH, or Tris. Concentrations of tryptone and yeast extract were limited to one-fourth of the original medium to decrease the Na^+ content (12).

Cells which were harvested in the late logarithmic phase of growth were used directly as K^+-loaded cells. The cells were collected by centrifugation, washed with 2 mM $MgSO_4$, and suspended as described for the individual experiments.

Transport experiments. Sodium movements were followed with $^{22}Na^+$ (8). Washed cells were suspended at 4 mg (dry weight) per ml in buffer with 20 mM $^{22}NaCl$ (0.8 μCi/ml) and incubated at 25°C for 60 min. At intervals, samples (0.2 ml) were filtered through membrane filters (pore size, 0.45 μm; Millipore Corp.) the filters were washed twice with the same buffer, and the radioactivity was measured with a liquid scintillation counter.

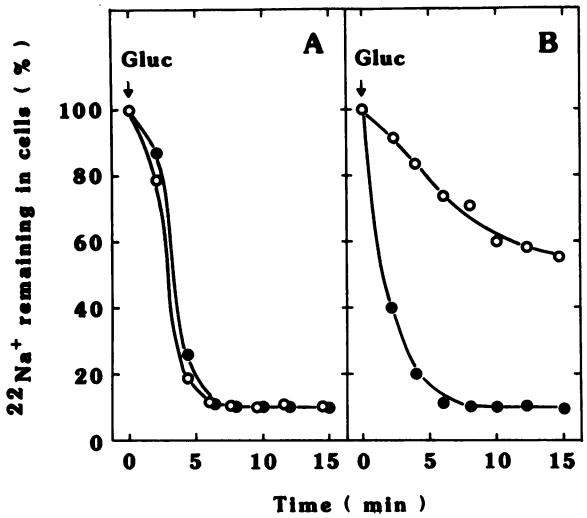

FIG. 1. Sodium extrusion from NaTY (A) and TrisTY (B) cells of strain ATCC 9790. Washed cells, which contained predominantly K^+ ions, were suspended at 4 mg of cells per ml in buffer containing 0.35 M potassium maleate and 0.05M potassium Tricine (pH 7.4 or 8.5). The pH was adjusted with maleic acid as needed. Then, 20 mM $^{22}NaCl$ (0.8 μCi/ml) was added, and the suspension was incubated at 25°C. After 60 min, the suspension was divided into aliquots, and 10 mM glucose (Gluc) was added at 0 min. Symbols: ●, pH7.4; ○, pH 8.5. The amounts represented as 100% were 50 (A) and 45 (B) nmol of Na^+ per mg of cells.

In pH shift experiments, cells were incubated in buffer (pH 8.5) containing 0.5 mM $^{22}NaCl$ (0.8 μCi/ml). After about 60 min, the medium pH was lowered to 6.0 by the addition of H_2SO_4.

Reagents. Radioactive materials were purchased from New England Nuclear Corp. Other reagents used were analytical grade.

Other. The membrane potential and the pH gradient of intact cells were calculated as described previously from the distribution of [^{3}H]tetraphenylphosphonium ion, [^{14}C]acetylsalicylic acid, and [^{14}C]methylamine (14).

Preparation of membrane vesicles and studies of ATP-dependent ^{22}Na uptake were carried out as described by Heefner and Harold (9). By functional criteria, the membrane vesicles were similar to the everted vesicles (16).

ATP-dependent changes of quinacrine fluorescence were measured with a Hitachi PMF4 fluorescence photometer as described elsewhere (9).

RESULTS

Sodium movements in intact cells. Figure 1 shows the extrusion of Na^+ from cells grown in either NaTY or TrisTY medium at two settings of the external pH. Washed cells (containing predominantly K^+) were incubated in buffer containing 400 mM K^+ [maleate-Tricine (Sigma Chemical Co.); Tricine is *N*-Tris(hydroxymethyl)methylglycine] and 20 mM $^{22}Na^+$ at the stated pH and in the absence of an energy source. After 60 min, the intracellular $^{22}Na^+$ concentration was equal to that in the external medium.

When cells were grown in NaTY medium, which contains about 120 mM Na^+ (NaTY cells), the addition of glucose elicited rapid expulsion of Na^+ against a concentration gradient of approximately 10-fold (Fig. 1A). Reagents that block or dissipate the proton circulation, such as *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of H^+-translocating ATPase, or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore, did not inhibit sodium extrusion (data not shown). These observations are consistent with the conclusion (7–10) that NaTY cells expel Na^+ ions by means of sodium ATPase.

When cells were grown on TrisTY medium, which contains about 5 mM sodium ions (TrisTY cells), and suspended at pH 8.5, sodium extrusion elicited by the addition of glucose was minimal (Fig. 1B). This result was not surprising, because sodium ATPase is not induced in TrisTY cells (12). At pH 7.4, however, rapid sodium extrusion was observed, a finding which raised the question of how these cells, without the sodium ATPase, carry out energy-dependent sodium extrusion.

Figure 2 shows the effects of CCCP and DCCD on the extrusion of Na^+ ions from TrisTY cells at two settings of the external pH. The minimal sodium extrusion seen at pH 8.5 was unaffected by the reagents (Fig. 2A). By contrast, rapid sodium extrusion at pH 7.4 was blocked (Fig. 2B), which suggested that the latter requires the generation of a proton motive force.

It is conceivable that the transport system which mediates sodium extrusion from TrisTY cells is sensitive to the cytoplasmic pH and that the inhibitory effects of proton conductors result from acidification of cytoplasmic pH. The parameters of the proton motive force were therefore determined as described in Materials and Methods. In TrisTY cells at pH 7.4, Δp was the sum of −20 mV (ΔpH) (pH_{in} 7.7) and −40 mV ($\Delta\psi$). At pH 8.5, Δp was the sum of +20 mV (ΔpH)(pH_{in} 8.2) and −20 mV ($\Delta\psi$). However, no detectable ΔpH and $\Delta\psi$ developed in the presence of DCCD and CCCP. Similar values were obtained with NaTY cells. As expected, the intracellular pH was much the same in all cases, but the total Δp was −60 mV at pH 7.4 and 0 mV at pH 8.5. It therefore seems unlikely that the inhibition of Na^+ extrusion by ionophores at pH 7.4 is due to cytoplasmic acidification.

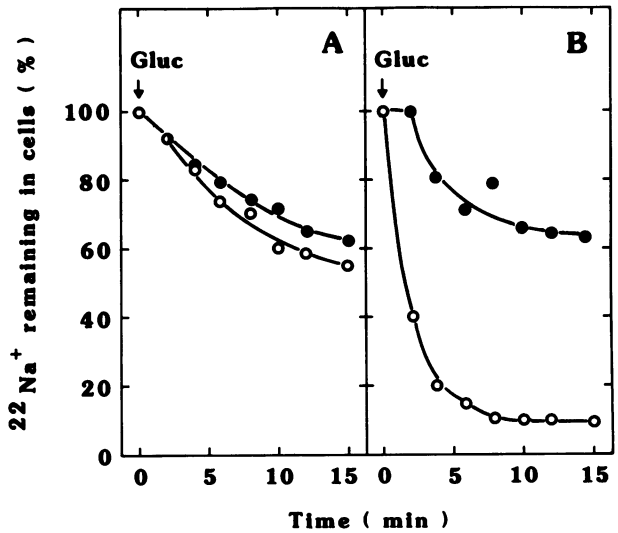

FIG. 2. Effect of inhibitors on sodium extrusion from TrisTY cells of strain ATCC 9790. Sodium extrusion was measured at pH 8.5 (A) or 7.4 (B) as described in the legend to Fig. 1. The cell suspension was divided into aliquots with additions as follows: ○, 10 mM glucose (Gluc) at 0 min; ●, 0.4 mM DCCD and 20 μM CCCP at −10 min and glucose at 0 min. The amounts represented as 100% were 46 nmol of Na^+ per mg of cells.

It is more likely that these reagents dissipate in proton motive force that drives sodium extrusion.

Figure 3 shows Na^+ extrusion from cells of the wild-type strain and of mutant AS25, both grown on KTY medium (KTY cells). Medium KTY contains only about 5 mM sodium ions and does not induce production of the sodium ATPase (12). Nevertheless, KTY cells at pH 7.4 extruded sodium ions by a process inhibitable by valinomycin (Fig. 3A). Inhibition of Na^+ extrusion was also observed with the addition of DCCD or CCCP. Sodium extrusion at pH 8.5 was minimal and was not blocked by these reagents (data not shown). AS25 is a mutant defective in the proton ATPase and in proton extrusion (15). When the mutant was grown in NaTY medium, the sodium ATPase was induced and mediated ionophore-resistant sodium extrusion (12, 13; data not shown). By contrast, when AS25 was grown in KTY medium, the cells had only minimal capacity for sodium extrusion at either pH 7.4 or 8.5 (Fig. 3B). It is known that AS25 cells grown in KTY medium also do not generate a proton motive force (15). Taken together, these observations indicate that DCCD-, CCCP-, or valinomycin-sensitive sodium extrusion at pH 7.4 (Fig. 1B, 2B, and 3A) is energized not by the sodium ATPase but by the proton motive force. It thus seems likely that *S. faecalis* cells produce a Na^+/H^+ antiporter.

Sodium movements driven by an imposed pH gradient. To verify the existence of a Na^+/H^+ antiporter, coupled exchange of Na^+ ions for protons must be demonstrated. Figure 4 shows Na^+ extrusion from TrisTY and NaTY cells in response to an applied pH gradient with the exterior acidic. Washed cells loaded with potassium ions were incubated in the absence of an energy source in 0.25 M K^+ (Tricine-maleate) buffer (pH 8.5) with 0.5 mM $^{22}NaCl$. The cells were pretreated with DCCD to preclude ATP synthesis in response to the subsequent imposition of a pH gradient. After 60 min of incubation, the extracellular pH was brought to 6.0 by the addition of H_2SO_4, thereby imposing a gradient of 2.5 pH units across the plasma membrane.

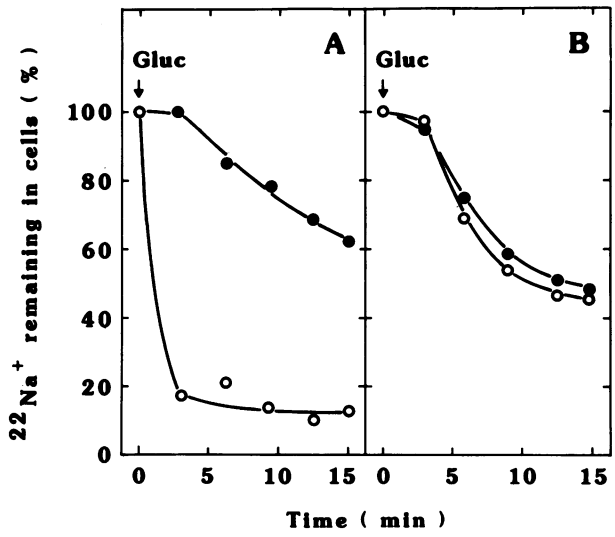

FIG. 3. Sodium extrusion from KTY cells of strains ATCC 9790 (A) and AS25 (B). Sodium extrusion was measured at pH 7.4 as described in the legend to Fig. 1. Symbols: ,○ 10 mM glucose added at 0 min; ●, 5 μM valinomycin added at −10 min and then glucose (Gluc) added. The amounts represented as 100% were 45 (A) and 48 (B) nmol of Na^+ per mg of cells.

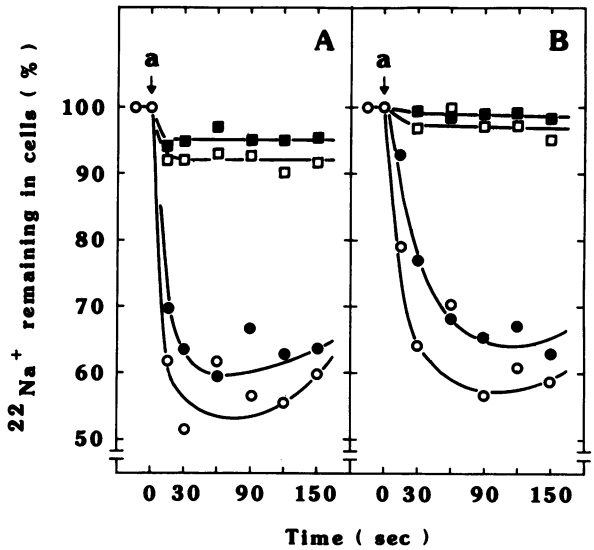

FIG. 4. Induction of sodium extrusion by pH shift. Washed cells were suspended at 4 mg of cells per ml in buffer containing 0.2 M potassium maleate, 0.05 M potassium Tricine (pH 8.5), and 0.5 mM $^{22}NaCl$ (0.8 μCi/ml) and incubated at 25°C for 60 min. The cell suspension was divided into aliquots with the addition of DCCD (0.4 mM) at −10 min and other chemicals at −5 min. (A) TrisTY cells. (B) NaTY cells. a, Addition of H_2SO_4 to drop the medium pH to 6.0; ○, no addition; ●, 10 μM valinomycin; □, 10 μM CCCP; ■, CCCP and valinomycin. The amounts represented as 100% were 5.1 (A) and 4.8 (B) nmol of Na^+ per mg of cells. The assay buffer contained 250 mM K^+, which contributed as much as 2.0 mM Na^+ according to the specifications of the manufacturer.

In TrisTY cells, which lack the sodium ATPase, transient sodium extrusion followed the addition of H_2SO_4 (Fig. 4A). Loss of $^{22}Na^+$ was unaffected by the addition of valinomycin (to dissipate any membrane potential) but was blocked by CCCP, CCCP plus valinomycin, and nigericin (Fig. 4A; nigericin data not shown). These results indicate that the pH gradient is required for Na^+ extrusion. Since sodium extrusion in response to a pH shift was also seen in potassium-depleted cells (data not shown), K^+ ions do not appear to participate in the sodium movements. Transient sodium extrusion in response to a pH gradient was also observed in NaTY cells (Fig. 4B). These cells do contain sodium ATPase, but the enzyme should be inactive because of the absence of glucose.

Is the Na^+/H^+ antiporter electroneutral or electrogenic? If it is electrogenic, imposition of a membrane potential (with the interior negative) should again elicit sodium extrusion, as Krulwich found with *Bacillus alcalophilus* (17). In the present study, when an artificial diffusion potential was imposed by adding valinomycin to K^+-loaded cells, $^{22}Na^+$ was not extruded but rather was accumulated by the cells. Heefner et al. (8, 10) suggested that this uptake represents electrogenic influx of Na^+ ions in response to the membrane potential, but it may also reflect the activity of the Na^+/H^+ antiporter. Addition of valinomycin allows K^+ to exit by exchange for protons and generates a pH gradient with the interior acidic. Na^+ ions are taken up by cells via a Na^+/H^+ antiporter. Attempts to distinguish between the two mechanisms were not successful.

Sodium uptake by membrane vesicles. Figure 5 shows ATP-driven uptake of $^{22}Na^+$ by membrane vesicles prepared from NaTY and TrisTY cells by the procedure of Heefner

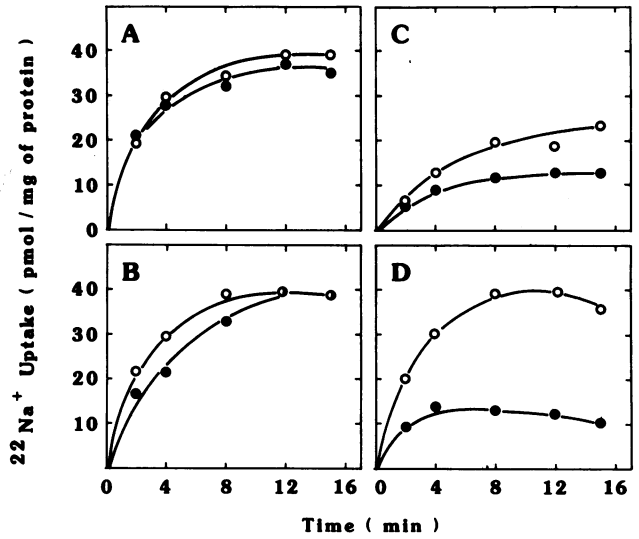

FIG. 5. Na^+ accumulation by membrane vesicles of NaTY and TrisTY cells of strain ATCC 9790. Na^+ accumulation was examined at pH 7.4 and 8.5 as described elsewhere (9) except that the assay was initiated by the addition of 5 mM ATP. CCCP (10 μM) was added at −10 min. (A) NaTY cells, pH 8.5. (B) NaTY cells, pH 7.4. (C) TrisTY cells, pH 8.5. (D) TrisTY cells, pH 7.4. Symbols: ○, ATP only; ●, ATP plus CCCP.

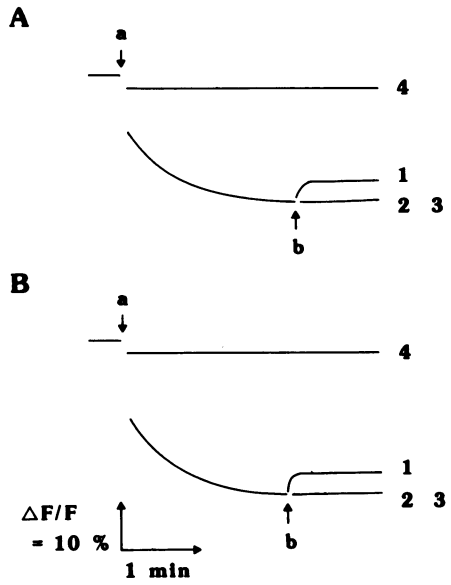

FIG. 6. Flurometric evidence for Na^+/H^+ antiporter. Preparations of membrane vesicles from NaTY or TrisTY cells of strain ATCC 9790 and the assay of fluorescence quenching were done as described elsewhere (9). Quinacrine (2.5 μM was added, followed by 2 mM ATP (a). Then, NaCl (1), KCl (2), or choline chloride (3) was added at a final concentration of 5 mM (b). CCCP (5 μM) was added before the addition of ATP (4). (A) Membranes of TrisTY cells (0.55 mg of protein per ml). (B) Membranes of NaTY cells (0.58 mg of protein per ml). F, Intensity of fluorescence.

and Harold (9). Vesicles from NaTY cells accumulated sodium ions by a process resistant to DCCD and CCCP at both pH 7.4 and 8.5 (Fig. 5A and B). The sodium ATPase is known to be induced in cells grown on NaTY and apparently energizes $^{22}Na^+$ uptake by the vesicles, as proposed by Heefner et al. (8–10).

Vesicles from TrisTY cells, which do not contain the sodium ATPase, behaved differently. Uptake of $^{22}Na^+$ at pH 8.5 was minimal; at pH 7.4 uptake was extensive but was blocked by DCCD and CCCP (Fig. 5C and D). I conclude that vesicles from TrisTY cells, like the intact cells, accumulate $^{22}Na^+$ with the aid of the proton motive force.

Finally, Na^+-dependent proton movements in membrane vesicles were examined by the quenching of quinacrine fluorescence. When membrane vesicles from TrisTY cells were incubated with ATP, quinacrine fluorescence declined, indicating proton influx via the proton ATPase. The addition of Na^+ ions induced some alkalinization of the vesicles; K^+ ions and choline did not (Fig. 6A). Sodium-dependent proton efflux was also seen in vesicles prepared from NaTY cells (Fig. 6B) but not in those prepared from a mutant deficient in sodium extrusion (6, 9) (data not shown). The results indicate that the exchange of H^+ for Na^+ does not involve the sodium ATPase and are consistent with the presence of a Na^+/H^+ antiporter.

DISCUSSION

Bacteria have evolved diverse mechanisms for the active extrusion of sodium ions. Secondary Na^+/H^+ antiporters are widely distributed, and some bacteria have recently been found to produce a primary sodium pump. In *Vibrio alginolyticus*, sodium extrusion is coupled directly to the respiratory chain (22). In *Klebsiella aerogenes*, sodium extrusion is linked to the decarboxylation of oxaloacetate (1).

For *S. faecalis*, Heefner and Harold demonstrated that sodium is expelled by means of a sodium ATPase (9). These authors also noted Na^+/H^+ antiport activity in some of their preparations, but they attributed it to proteolytic damage to sodium ATPase molecules (8–10). The results reported here indicate that the antiport activity is not artifactual but reflects the presence of a native Na^+/H^+ antiporter in both membrane vesicles and intact cells.

It is noteworthy that Na^+/H^+ antiport activity was observed in both TrisTY cells, which lack the sodium ATPase, and NaTY cells, in which the enzyme is present. Reagents that dissipate the proton motive force inhibited sodium extrusion from TrisTY cells but not from NaTY cells, because in the former the Na^+/H^+ antiporter is the chief pathway present, whereas the latter contain the sodium pump as well. The sodium ATPase is known to be inducible (12, 13), but the Na^+/H^+ antiporter appears to be constitutive. The existence of dual systems has been suggested for calcium extrusion in streptococci (2, 11).

Is the Na^+/H^+ antiporter electrogenic or electroneutral? The sodium gradient ($Na^+_{out} > Na^+_{in}$) generated by TrisTY cells was greater than 10-fold (Fig. 1B). The pH gradient under these conditions was as low as −20 mV, not sufficient to account for sodium extrusion by electroneutral Na^+/H^+ exchange. Moreover, sodium extrusion was blocked by valinomycin, which suggests a requirement for $\Delta\psi$ (Fig. 3A). I therefore favor the hypothesis that the Na^+/H^+ antiporter is electrogenic.

Why does *S. faecalis* require two systems for sodium extrusion? *S. faecalis* lives in an environment of fluctuating pH (pH 6 to 10). At acidic pH, the generation of a proton motive force is large enough to drive a Na^+/H^+ antiporter and other proton-linked transport systems. The proton motive force is drastically decreased at pHs above 8 (14). At pH 10, the proton potential across the plasma membrane turns positive by 1 U or more (unpublished results). The Na^+/H^+

antiporter cannot operate under these conditions, and induction of the sodium ATPase is required for the growth. There is a good evidence that the sodium ATPase is induced in media rich in sodium and also in the presence of reagents that dissipate the proton motive force (12, 13), presumably because under these conditions the antiporter is not sufficient to exclude Na^+ from the cytoplasm. Thus, the existence of two systems for sodium extrusion allows the organism to cope with an environment subject to fluctuations in both ionic composition and pH.

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