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Non-Proton-Motive-Force-Dependent Sodium Efflux from the Ruminal Bacterium *Streptococcus bovis*: Bound versus Free Pools

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Growing cells of *Streptococcus bovis* JB1 had a sodium content of 1,125 nmol/mg of protein and, based on a ratio of cell volume to protein of 4.3 μl/mg, the apparent intracellular sodium concentration was more than 240 mM. Much of this sodium could not be removed by water washing even if cells were boiled or treated with the pore-forming ionophore, gramicidin, but it could be exchanged for potassium. Stationary cultures had a 2.6-μl volume per milligram of protein and a total sodium content of 410 mM. When stationary cultures were energized with glucose at pH 6 to 8, sodium (more than 200 mM) was expelled within 2 min, and it appeared that growing cells had a very small pool of free intracellular sodium. Sodium-proton antiport activity could not be demonstrated with a sodium pulse, and the protonophore SF6847, valinomycin, and the H^+-ATPase inhibitor dicyclohexylcarbodiimide (DCCD) had little effect on sodium efflux, even though these inhibitors greatly reduced the proton-motive force. SF6847, valinomycin, and DCCD had little effect on intracellular ATP, but iodoacetate, an inhibitor of glycolysis, decreased ATP as well as sodium efflux. Stationary cells from sodium-deficient medium expelled little sodium after glucose addition and had 35% more ATP than stationary cells which were grown in sodium medium and expelled sodium. An artificial electrochemical gradient of sodium was able to drive ATP synthesis in stationary cells, and this ATP formation was not sensitive to DCCD. These results indicated that bacteria could have a significant pool of bound sodium and that sodium expulsion from *S. bovis* was directly coupled to ATP hydrolysis.

The maintenance of low intracellular sodium appears to be a feature of most living organisms, and bacteria can use a variety of methods to expel sodium including ATPases, respiratory chains, membrane-bound decarboxylases and sodium-proton antiporters (14). Because inwardly directed chemical gradients of sodium can be used to drive solute transport (13), motility (3), and ATP synthesis (5), sodium expulsion can be a critical feature of membrane bioenergetics and growth. The rumen is a sodium-rich habitat, and many ruminal bacteria have a requirement for sodium (1).

Streptococcus bovis is a rapidly growing rumen bacterium that proliferates when animals are fed large amounts of starch (12), and recent work demonstrated that it was only able to transport neutral amino acids if sodium was present (13). A chemical gradient of sodium drove uptake in deenergized cells and membrane vesicles (13), but other studies indicated that growing cells had a large pool of cellular sodium (11). Because the kinetics of sodium-dependent transport appeared to contradict measurements of intracellular sodium, we decided to investigate the process of sodium expulsion from *S. bovis*. Results indicated that much of the sodium in whole cells was bound. Free sodium was expelled by a mechanism that (i) was independent of the proton-motive force, (ii) did not occur in antiport with protons, and (iii) was driven directly by ATP hydrolysis.

MATERIALS AND METHODS

Organism and growth conditions. *S. bovis* JB1 was used, and this strain is characteristic of the species (12). Cultures were grown anaerobically in medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 600 mg of cysteine hydrochloride, 4,000 mg of Na_2CO_3, 1,000 mg of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), and 500 mg of yeast extract. When the organism was grown in sodium-deficient medium, sodium salts were replaced by potassium salts. Sodium contamination from Trypticase and yeast extract was less than 1.6 mM. Glucose was added to the basal medium (final concentration, 30 mM) as a separate solution. The medium was adjusted to pH 6.7, and incubations were at 39°C.

Measurement of sodium, potassium, and sodium binding. Intracellular concentrations of sodium and potassium were determined in stationary and exponentially growing cultures after various washing procedures. Cells (0.6 mg of protein) were boiled (15 min), treated with gramicidin (40 μM), or washed twice with 100 mM KCl. Cells were washed twice with distilled water after each treatment and centrifuged (13,000 × *g*, 5 min) through silicon oil (Dexter Hysol 550; Hysol Co., Olean, N.Y.). Treated cells were compared with water-washed cells or controls which were not washed. Silicon oil was removed, and the cell pellets were dissolved in 3 N HNO_3 for at least 24 h. Sodium and potassium were analyzed with a Perkin-Elmer (Norwalk, Conn.) atomic absorption spectrophotometer, and values were corrected for extracellular contamination.

Sodium-free cells for sodium-binding experiments were prepared by treating stationary-phase cells with gramicidin (40 μM) and washing twice with 100 mM potassium chloride and twice with distilled water. These sodium-free cells (3.9 mg of protein) were then added to 100 mM Tris hydrochloride (pH 7.0) which contained 0 to 40 mM NaCl. Changes in free-sodium concentration were measured at 39°C with a combination sodium electrode (Microelectrodes, Inc., Londonderry, N.H.). The final volume of the assay was 2.5 ml.

Sodium efflux measurements. Stationary cultures (160 ml, optical density at 600 nm of 2.0) were centrifuged (7,500 × *g*, 5 min, 4°C), suspended in 4 ml of fresh medium, and stored

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on ice. Concentrated cells (10 mg of protein per ml) were diluted fivefold with 350 mM potassium maleate–50 mM tricine (pH 7.0), and sodium efflux was initiated by the addition of 5 mM glucose. Ionophores and metabolic inhibitors were added 15 min prior to glucose. Changes in the extracellular sodium concentration were measured with a combination sodium electrode at 39°C.

Proton-motive force determinations. Internal pH was determined by an acid distribution method (10). Cells (0.19 mg of protein per ml) were incubated in the presence of [7-^{14}C]benzoate (0.4 μCi), [1,2-^{14}C]-polyethylene glycol (0.8 μCi), or $^{3}H_2O$ (4.0 μCi) for 5 min and then centrifuged through silicon oil (Dexter Hysol 550; Hysol Co.) in a microcentrifuge (13,000 × *g*, 5 min). Supernatant samples (20 μl) were removed, and bottoms of tubes containing cell pellets were removed with dog nail clippers after freezing. Pellets and supernatants were dissolved in aqueous-compatible scintillation fluid. The internal volume was estimated from the difference in specific activity of $^{3}H_2O$ and [^{14}C]polyethylene glycol. The membrane potential ($\Delta\psi$) was determined from the distribution of the lipophilic cation [phenyl-^{14}C]tetraphenylphosphonium bromide (TPP^+). Cells (0.19 mg of protein per ml) were incubated 5 min with 0.5 μCi of TPP^+ and processed as described above. Nonspecific binding was estimated from TPP^+ binding to nonenergized cells which had been treated with 10 μM valinomycin plus 10 μM nigericin.

ATP determinations. Cells (approximately 2.2 mg of protein in 1.0 ml) were extracted for 20 min with 0.5 ml of ice-cold, 14% perchloric acid which contained 9 mM EDTA. Macromolecules were removed by centrifugation (13,000 × *g*, 5 min, 22°C), and the supernatant (1 ml) was neutralized with 0.5 ml of KOH-$KHCO_3$ (1 M each, 0°C). Potassium perchlorate was removed by centrifugation (13,000 × *g*, 5 min, 22°C), and the extract was assayed for ATP by the firefly luciferase method (8). Neutralized extracts were diluted 50-fold with 40 mM Tris containing 2 mM EDTA, 10 mM $MgCl_2$, and 0.1% bovine serum albumin (pH 7.75). The luciferase reaction was initiated by adding 100 μl of a purified luciferine-luciferase mix to 100 μl of diluted extract according to the recommendations of the supplier (Sigma Chemical Co., St. Louis, Mo.). Light output was immediately measured with a luminometer (model 1250, LKB Instruments, Inc., Gaithersburg, Md.) using ATP as a standard.

ATP synthesis. Stationary cells (80 ml, 2.0 optical density at 600 nm) from sodium-deficient medium were centrifuged (7,500 × *g*, 5 min, 4°C), washed three times with morpholineethanesulfonic acid (MES; 100 mM, adjusted to pH 6.0 with choline base), and suspended in 4 ml of MES. Assays were conducted with 20-fold-diluted cells which were suspended in MES buffer, and potassium efflux was initiated by the addition of valinomycin (final concentration, 10 μM). In assays where reduced potassium efflux was desired, potassium chloride (final concentration, 6 mM) was added to the buffer. Sodium-dependent ATP formation was elicited by the addition of 100 mM sodium chloride, and samples (1 ml) were assayed for ATP as described above.

Materials. Radiolabeled compounds were obtained from Amersham Corp., Arlington Heights, Ill. The protonophore 3,5-di-tert-4-hydroxy-benzilidene malonitrile (SF6847) was kindly provided by W. N. Konings, University of Groningen, the Netherlands. All other chemicals were obtained from commercial sources.

TABLE 1. Sodium and potassium contents of *S. bovis* exponential or stationary cells which were treated with various washing procedures[a]

Treatment for exponential or stationary cells	Sodium concn		Potassium concn	
	nmol/mg of protein	mM[b]	nmol/mg of protein	mM[b]
Exponential cells				
None	1,125	245	3,035	661
Water wash	417	91	1,451	122
Boiled + water wash	450	98	31	7
Gramicidin + water wash	353	77	46	10
KCl + water wash	73	16	1,515	330
Stationary cells				
None	1,063	410	1,968	759
None[c]	18	7	1,525	615
Water wash	1,020	390	201	84
Gramicidin + water wash	178	70	62	25
Gramicidin + KCl + water wash	0	0	878	367

[a] See Materials and Methods for details.
[b] Based on a ratio of cell volume to protein of 4.3 μl/mg for growing cells and 2.6 μl/mg of protein for stationary cells.
[c] Cells were grown in a sodium-deficient medium (less than 1.6 mM sodium).

RESULTS

Bound versus free sodium. When *S. bovis* was grown in medium containing 94 mM sodium, cells had a total of 1,125 nmol of sodium per milligram of protein. Because the ratio of intracellular volume to protein was 4.3 μl/mg, the apparent intracellular concentration of sodium was 245 mM. However, sodium was retained even if cells were boiled in water or treated with the channel-forming ionophore, gramicidin (Table 1). Since the remaining sodium could be removed only after repeated washing with potassium chloride, it appeared that much of the cellular sodium was tightly bound to the cell envelope. The potassium concentration was greater than 600 mM, but more than 95% was removed by boiling or gramicidin treatment.

Stationary cells had 2.6 μl of intracellular volume per milligram of protein and an even higher total sodium concentration (Table 1). Because stationary and growing cells had similar amounts of sodium after gramicidin treatment, it appeared that the stationary cells had accumulated a large pool (>150 mM) of free intracellular sodium. When cultures were grown in sodium-deficient medium, stationary cells contained less than 20 nmol of sodium per milligram of protein (7 mM) and only 15% more potassium than in growing cells (759 mM).

When stationary cells were treated with gramicidin, washed with potassium chloride, and then washed with distilled water, virtually all of the sodium was removed (Table 1). If these sodium-free cells were added to Tris buffer containing sodium chloride, sodium binding could be measured with a sodium electrode. Preliminary experiments indicated that 20 mM was needed for saturation. Based on a maximal change of 1.6 mM sodium (Fig. 1) and a cell concentration of 3.9 mg of protein per 2.5 ml, the gramicidin-treated cells bound 1,026 nmol of sodium per milligram of protein.

Sodium efflux. When stationary cultures of *S. bovis* were incubated in sodium-free, potassium maleate-tricine buffer (pH 7.0), little sodium was lost, but the addition of glucose

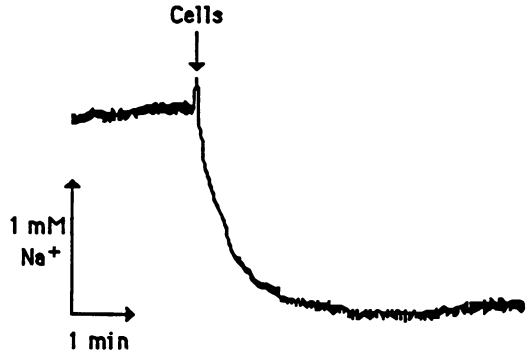

FIG. 1. Binding of sodium to stationary cells which were treated with gramicidin (40 μM), washed twice with 100 mM KCl, and twice washed with distilled water. Cells (3.9 mg of protein) were suspended in 100 mM Tris (pH 7.0, 2.5 ml) containing 20 mM NaCl.

caused an immediate efflux of sodium (Fig. 2a) and a decrease in extracellular pH (0.1 U because of lactate production). This small decline in pH did not interfere with the response of the electrode. There was little change in the extent or rate of sodium efflux by glucose-energized cells if extracellular pH varied from 8.0 to 6.0 or if 200 mM potassium lactate was added to the assay buffer. However, if pH was less than 6.0, little sodium efflux or acidification (lactate production) was noted. Sodium efflux was also observed in potassium-free buffer (100 mM Tris hydrochloride, pH 7.0), and the addition of potassium did not affect efflux.

When glucose-energized cells were treated with the protonophore SF6847 (Fig. 2b), the ionophore valinomycin (Fig. 2c), or the F_0F_1 H^+-ATPase inhibitor dicyclohexylcarbodiimide (DCCD; Fig. 2d), there was little effect on either the rate or extent of sodium efflux. These inhibitors caused large reductions in Δp but had minimal effects on intracellular ATP (Table 2). Increasing amounts of iodoacetate inhibited sodium efflux (Fig. 2e through g), and this inhibition was correlated with a decrease in ATP (Fig. 3).

If *S. bovis* was incubated in sodium-deficient medium (less than 1.6 mM sodium), there was no change in maximum growth rate (1.4/h) and glucose-dependent sodium efflux from stationary cells could not be elicited. However, if these same cells were incubated with 100 mM sodium chloride for 1 h, cellular sodium increased to 475 mM and sodium efflux was restored (data not shown). When DCCD-treated cells were energized with a small dose of glucose (5 mM), there was a rapid increase in ATP (Fig. 4). The ATP content of cells which were grown in sodium-deficient medium and did not expel sodium reached a maximum of 6.2 mM, while cells which were grown in 94 mM sodium and expelled sodium contained less than 4.5 mM ATP.

TABLE 2. Effect of ionophores and DCCD on proton-motive force (Δp) and intracellular ATP concentrations

Treatment for energized or nonenergized cells[a]	ZΔpH (mV)	Δψ (mV)	Δp (mV)	ATP[b] (mM)
Nonenergized	−3	36	33	0.15
Glucose-energized	55	76	131	2.81
SF6847	4	12	16	2.44
Valinomycin	39	26	65	2.30
DCCD	−7	18	11	3.52

[a] Cells were energized with 5 mM glucose. Ionophores (10 μM) and DCCD (0.25 μmol/mg of protein) were added 15 min prior to glucose addition.
[b] Cells (2.0 mg of protein per ml) for ATP determinations were sampled 1.5 min after glucose addition.

Nonenergized cells that were suspended in potassium maleate-tricine buffer retained a large pool of intracellular sodium (>150 mM) for a considerable period of time (more than 20 min), even in the presence of the protonophore SF6847 (data not shown). If nonenergized cells were washed twice with 100 mM potassium chloride to remove virtually all of the intracellular sodium and incubated in lightly buffered medium (2 mM glycyl-glycine, pH 7.0), a pulse dose of sodium (20 mM) did not cause extracellular acidification, even though our detection limit was as small as 0.005 pH units (data not shown). Neither of these results were compatible with the presence of a sodium-proton antiporter.

ATP synthesis. When stationary cells were suspended in potassium-free buffer and treated with valinomycin to establish an artificial potassium diffusion potential (Δψ), there was a 20-fold increase in ATP which could be eliminated by DCCD (Fig. 5). When cells were incubated in buffer containing 6 mM potassium (less Δψ), little ATP synthesis was observed. However, if 100 mM sodium chloride was added, there was an increase in ATP and this increase was not sensitive to DCCD. When DCCD was present, the Δψ would not have been dissipated by the F_0F_1 H^+-ATPase, and ATP formation continued for a longer period of time. The sodium ATPase was specific for sodium and could not utilize lithium or choline (data not shown).

DISCUSSION

Since the rumen is a sodium-rich environment, it is not surprising that *S. bovis* possesses sodium-linked transport mechanisms (13). However, the uptake of amino acids by an

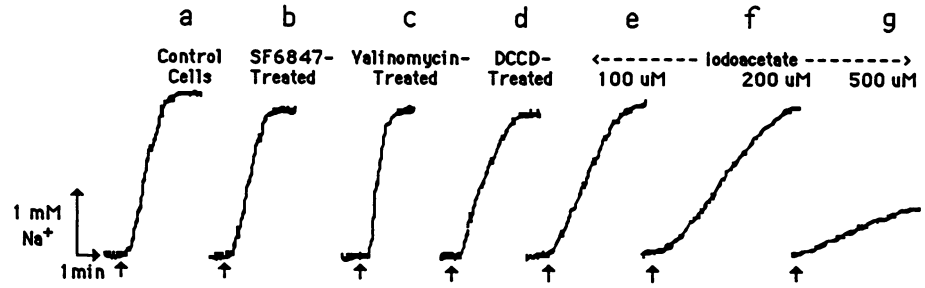

FIG. 2. Effect of metabolic inhibitors on sodium efflux from glucose-energized cells. Cells (2.0 mg of protein per ml) were suspended in 2.5 ml of 350 mM potassium maleate–50 mM Tricine (pH 7.0), treated 15 min with inhibitor (SF6847, 10 μM; valinomycin, 10 μM; DCCD, 0.25 μmol/mg of protein; iodoacetate, as specified), and energized with 5 mM glucose (arrow).

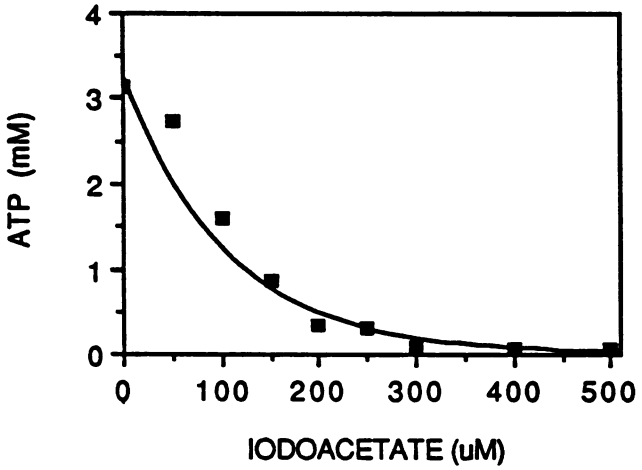

FIG. 3. Effect of iodoacetate on ATP synthesis by glucose-energized cells. Cells (2.2 mg of protein per ml) were extracted 2 min after the addition of 5 mM glucose.

inwardly directed sodium gradient (13) was difficult to reconcile with the observation that growing cells maintained a reversed sodium gradient where the concentration was approximately 2.7-fold higher inside than outside (11). This apparent discrepancy could be explained by the large amounts of bound sodium (Table 1).

Since water washing removed almost 60% of the total sodium, loosely associated capsular material could have contributed to sodium binding. The sodium which remained after water washing may have been associated with more tightly bound anionic components (e.g., peptidoglycan and teichoic acids). Because growing cells contained 1,125 nmol of sodium per milligram of protein (Table 1) and sodium binding was 1,026 nmol/mg of protein (Fig. 1), it appeared that less than 10% of the total sodium in growing cells was free. These results demonstrated that total sodium content was not an accurate measure of the free intracellular concentration. Recent experiments indicated that a ruminal peptostreptococcus also had a very high sodium content (2).

Stationary cells accumulated sodium which was expelled only after they were energized with glucose. Because

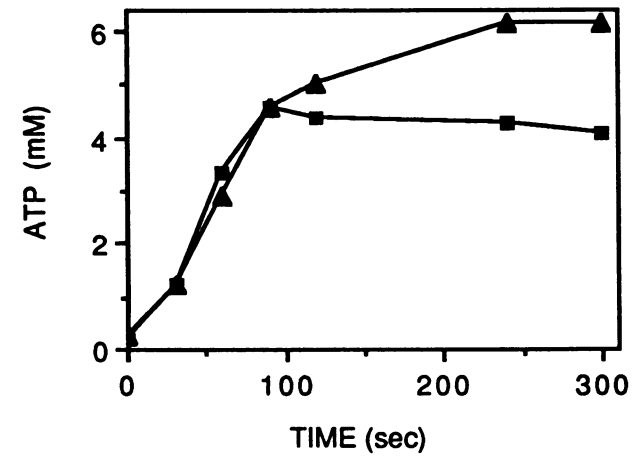

FIG. 4. ATP synthesis by cells grown in the presence of potassium (▲) or sodium (■). Cells (2.2 mg of protein per ml) were treated with DCCD (0.25 μmol/mg of protein) 15 min prior to glucose addition (5 mM).

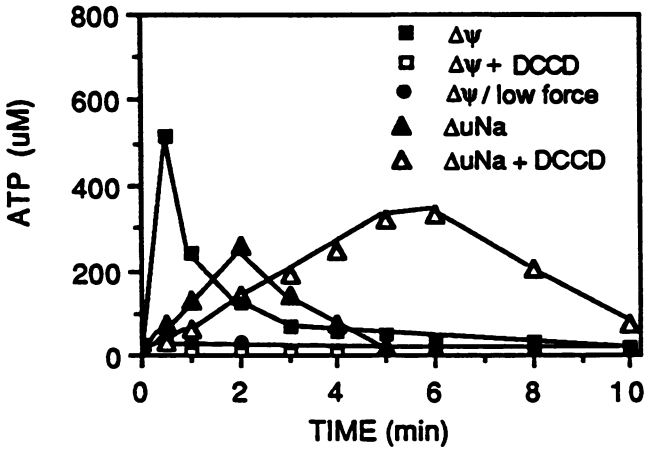

FIG. 5. ATP synthesis driven by an artificial membrane potential (Δψ), an electrochemical gradient of sodium (ΔuNa), or both. Cells grown in sodium-deficient medium (0.4 mg of protein per ml) were incubated in 100 mM MES (pH 6.0), and valinomycin (10 μM) was added at 0 min. Potassium chloride (6 mM) was added to reduce the membrane potential, and sodium chloride (100 mM) was added to create a sodium gradient. Cells were treated with DCCD (0.25 μmol/mg of protein) 15 min prior to valinomycin addition.

SF6847, valinomycin, and DCCD had no effect on sodium expulsion, even though they greatly reduced the Δp, proton efflux was not observed after a sodium pulse, and because SF6847 did not accelerate sodium loss from nonenergized cells, it appeared that sodium efflux was not mediated by a sodium-proton antiporter. *Streptococcus faecalis* has an electrogenic sodium-proton antiporter which is Δp dependent (6).

SF6847, valinomycin, and DCCD had little effect on either sodium efflux or ATP, but iodoacetate decreased both sodium expulsion and ATP. The role of ATP hydrolysis in sodium efflux was further supported by the observation that cells which were grown in sodium-deficient media contained 35% more ATP than ones which were grown in sodium-containing medium (Fig. 4). Cells which were grown in sodium-deficient medium contained little sodium and would not have used ATP to expel sodium.

Membrane-bound ATPases are theoretically reversible, but at least 175 mV of Δψ was required to drive ATP synthesis in *Streptococcus lactis* (9). When *S. bovis* was incubated in MES choline buffer and treated with valinomycin, Δψ caused by potassium efflux was theoretically more than 200 mV and there was a 20-fold increase in ATP (Fig. 5). Since ATP synthesis was DCCD sensitive, it appeared that the F_0F_1 H^+-ATPase was probably responsible for ATP formation. If 6 mM extracellular potassium was present, Δψ decreased to 120 mV and ATP synthesis was abolished.

An artificial Δψ of 120 mV was unable to drive the F_0F_1 H^+-ATPase, but ATP synthesis was observed after the addition of 100 mM sodium (Fig. 5). Because these cells were grown in sodium-deficient medium and had a very low concentration of intracellular sodium, a chemical gradient of sodium appeared to provide an additional driving force for ATP generation. Since sodium-dependent ATP synthesis was not sensitive to DCCD, it seemed that sodium translocation was mediated by an ATPase which was distinct from the F_0F_1 H^+-ATPase.

Heefner et al. showed that everted vesicles of *S. faecalis* could use ATP to accumulate sodium (4, 7), but in this

organism sodium efflux (i) was induced by sodium, (ii) was active only at alkaline pH, (iii) required extracellular potassium, and (iv) appeared to be mediated by potassium-sodium ATPase (*Ktr*II). The *S. bovis* sodium ATPase was constitutive, was active from pH 6 to 8, and did not require extracellular potassium. Although the sodium ATPase of *S. bovis* was reversible, it is probably not involved in ATP formation under physiological conditions.

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