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Volume Regulation in *Mycoplasma gallisepticum*: Evidence that Na^+ Is Extruded via a Primary Na^+ Pump

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The primary extrusion of Na^+ from *Mycoplasma gallisepticum* cells was demonstrated by showing that when Na^+-loaded cells were incubated with both glucose (10 mM) and the uncoupler SF6847 (0.4 μM), rapid acidification of the cell interior occurred, resulting in the quenching of acridine orange fluorescence. No acidification was obtained with Na^+-depleted cells or with cells loaded with either KCl, RbCl, LiCl, or CsCl. Acidification was inhibited by dicyclohexylcarbodiimide (50 μM) and diethylstilbesterol (50 μM), but not by vanadate (100 μM). By collapsing $\Delta\psi$ with tetraphenylphosphonium (200 μM) or KCl (25 mM), the fluorescence was dequenched. The results are consistent with a $\Delta\psi$-driven uncoupler-dependent proton gradient generated by an electrogenic ion pump specific for Na^+. The ATPase activity of *M. gallisepticum* membranes was found to be Mg^{2+} dependent over the entire pH range tested (5.5 to 9.5). Na^+ (>10 mM) caused a threefold increase in the ATPase activity at pH 8.5, but had only a small effect at pH 5.5. In an Na^+-free medium, the enzyme exhibited a pH optimum of 7.0 to 7.5, with a specific activity of 30 ± 5 μmol of phosphate released per h per mg of membrane protein. In the presence of Na^+, the optimum pH was between 8.5 and 9.0, with a specific activity of 52 ± 6 μmol. The Na^+-stimulated ATPase activity at pH 8.5 was much more stable to prolonged storage than the Na^+-independent activity. Further evidence that two distinct ATPases exist was obtained by showing that *M. gallisepticum* membranes possess a 52-kilodalton (kDa) protein that reacts with antibodies raised against the β-subunit of *Escherichia coli* ATPase as well as a 68-kDa protein that reacts with the anti-yeast plasma membrane ATPase antibodies. It is postulated that the Na^+-stimulated ATPase functions as the electrogenic Na^+ pump.

It was previously postulated that in *Mycoplasma gallisepticum*, Na^+ is extruded from the cells by the combined action of a proton ATPase and an Na^+/proton antiporter (10, 11). Nevertheless, as Na^+ movement in this organism was found to be neither driven nor inhibited by the collapse of the electrochemical gradient of H^+, it was suggested that in this organism Na^+ is extruded by an electrogenic primary Na^+ pump (27).

Four distinct primary Na^+ transport systems have been observed in bacteria. These include respiration-driven Na^+ pumps (29, 30), Na^+-translocating decarboxylases (3), and Na^+-transporting ATPases (2, 5, 6). The unifying feature of these primary mechanisms is the resistance of Na^+ transport to uncouplers (19). It was suggested that ATPases play a central role in cell volume regulation in mycoplasmas (32). In both mycoplasmas and *Spiroplasma* species (belonging to the class *Mollicutes*, the wall-less procaryotes [17]), a membrane protein that interacts specifically with antibodies generated against the β-subunit of *Escherichia coli* ATPase has been described (24, 33). This observation was taken to suggest that mycoplasmas, like most bacteria, possess a proton-translocating F_1F_0-ATPase (24). Nevertheless, only 44% of the total membrane-bound ATPase activity of *M. gallisepticum* could be immunoprecipitated by the anti-*E. coli* β-subunit antiserum, suggesting the presence of a second ATPase (33).

Recently, a five-subunit ATPase was isolated from the *Acholeplasma laidlawii* membrane and proposed to act as an Na^+ pump (7, 9). In *M. mycoides*, Na^+ extrusion against a concentration gradient was postulated to be mediated by an Na^+-ATPase (2).

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In the present study, we document a primary Na^+ pump in *M. gallisepticum*, show that this organism has an Na^+-stimulated ATPase activity, and suggest that this ATPase mediates translocation of Na^+.

MATERIALS AND METHODS

Growth and harvesting of organisms. *Mycoplasma gallisepticum* A5969, *M capricolum* (Kid), and *Acholeplasma laidlawii* (OR) were grown at 37°C for 18 to 26 h in a modified Edward medium (18) containing 4% horse serum. Growth was monitored by measuring the A_{640}, and cells were harvested at the mid-exponential phase of growth (A_{640} 0.2 to 0.25) by centrifugation at 12,000 × *g* for 10 min. The cells were washed once with and suspended in isoosomotic solutions of either sodium chloride (0.25 M), sucrose (0.5 M), glycylglycine (0.25 M), or choline chloride (0.25 M).

Preparation of membranes. Membranes were obtained from cells lysed either by the glycerol lysis procedure (18) or by ultrasonic treatment (22). For glycerol lysis, cells (1 mg of protein per ml) were suspended in 5 ml of a 2 M glycerol solution and incubated at 37°C for 10 min. The cells were then rapidly injected into 250 ml of deionized water containing 5 mM Tris hydrochloride (pH 8.5) and 1.0 mM dithiothreitol (DTT) and incubated at 37°C for 15 min. For ultrasonic treatment, cells (1 mg of protein per ml) were ultrasonically treated for 3 min at 0°C in a W-350 Heat Systems sonicator operated at 50% duty cycles at 200 W. In both procedures, the lysed cell suspensions were centrifuged at low speed (3,900 × *g* for 5 min) to remove unbroken cells, and the membranes were collected by centrifugation at 34,000 × *g* for 30 min. Protein in the cell and membrane preparations was determined by the method of Lowry et al. (13).

Measurement of intracellular water volume. To determine intracellular volume, cells were incubated in 2.4 ml of a solution containing 250 mM Tris-MOPS (morpholinopropanesulfonic acid) buffer, adjusted to the appropriate pH, ^3H_2O (4.3 μCi/ml), [^{14}C]polyethylene glycol (PEG; 0.1 μCi/ml), and a 10-fold excess of unlabeled PEG. In most experiments, 10 mM glucose was added. After incubation at 37°C for 15 min, 1-ml samples were pipetted onto the surface of silicone oil (0.3 ml) in 1.5-ml plastic microfuge tubes and centrifuged at 12,800 × *g* for 2 min. Under these conditions, the cells pass through the silicone oil, forming a pellet at the bottom of the tube, while the aqueous phase remains on top of the silicone oil (21). After samples of the aqueous phase were withdrawn for determination of radioactivity, the aqueous and silicone phases were removed by suction, the tips of the centrifuge tubes (containing the cell pellets) were cut off, and radioactivity was counted. The ^{3}H counts were taken as a measure of total pellet water; since PEG cannot enter the cells, the [^{14}C]PEG counts were taken as a measure of the extracellular space. In most experiments, the PEG space was approximately 20% of the total pellet water. The intracellular water volume was calculated by subtracting the PEG space value from the total water space value.

Measurements of ΔpH and Δψ. For the measurement of ΔpH, the distribution of either ^{14}C-labeled benzoic acid (0.04 μCi/ml) or ^{14}C-labeled methylamine was determined (21). For the measurement of membrane potential, the distribution of ^{3}H-labeled tetraphenylphosphonium ([^{3}H]TPP; 0.03 μCi/ml) was determined (21). In brief, cells (1.5 mg of cell protein per ml) were incubated in a solution containing 250 mM NaCl, 10 mM glucose, and 25 mM Tris-MOPS for 10 min at 37°C. Samples (1 ml) were then layered onto the surface of silicone oil in the 1.5-ml microfuge tubes and treated as described above. Intracellular and extracellular concentrations of labeled benzoic acid, methylamine, or TPP were determined, and the ΔpH and Δψ were calculated (21).

Acridine orange fluorescence measurements. Primary sodium-pumping activity was assayed by monitoring the membrane potential-driven uptake of protons in cells in the presence of an uncoupler. The formation and maintenance of ΔpH due to the uptake of protons was estimated by determining the fluorescence quenching of the dye acridine orange (3, 14, 26). Fluorescence studies were done in a Perkin-Elmer spectrofluorometer with excitation of 492 nm and emission at 530 nm. Cells (final concentration, 30 μg of protein per ml) suspended in 2.5 ml of buffer (reaction medium, consisting of 250 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 8.5], and 5 μM acridine orange) were made permeable to protons by the addition of 0.4 μM SF6847 (provided by Y. Shahak, Weismann Institute). Fluorescence quenching was initiated by the addition of an energy source (10 mM glucose) and followed at 37°C.

$^{22}Na^+$ efflux. $^{22}Na^+$ efflux measurements were performed with preloaded *M. gallisepticum* cells, and efflux was initiated by the addition of glucose. Cells (6 mg/ml) were loaded with $^{22}Na^+$ by incubation for 2 h at 37°C in 0.4 M sucrose solution containing the desired concentration of NaCl, 25 mM Tris-MOPS buffer (pH 7.5), and 3.0 μCi of $^{22}Na^+$ per ml. The loaded cells were diluted 1:200 into a reaction mixture containing 110 ml of either 0.5 M sucrose or an isoosmotic sucrose-NaCl solution (adjusted to pH 6.5, 7.5, or 8.0) in the presence and absence of 10 mM glucose. The reaction mixtures were incubated at 32°C, and at the indicated time intervals, 10-ml portions were withdrawn and filtered through fiberglass filters (25 mm GF/C; Whatman, Inc.) under negative pressure (filtration time, 3 s). The filters were washed twice with a 10-ml volume of cold 0.25 M NaCl, and the radioactivity retained on the filters was determined. Over 95% of the cells were retained on the filters, as judged from measuring the retention of [^{14}C]palmitate-labeled cells.

Measurement of ATPase activity. Two methods were used for determining ATPase activity. In both methods, ATPase activity was determined in *M. gallisepticum* membranes by measuring the release of P_i. In the colorimetric assay, ATPase activity was assayed in a reaction mixture (1.0 ml) containing 100 μg of membrane protein and 50 mM Tris-MOPS buffer with or without NaCl (10 mM), 4.0 mM $MgCl_2$, 4.0 mM Tris, or sodium ATP (vanadate free). The reaction was carried out at 37°C for various periods of time and stopped by the addition of 1 ml of cold 20% trichloroacetic acid to each tube. The tubes were then centrifuged at 3,000 × *g* for 5 min, and phosphate was determined in the deproteinized supernatant by a modified method of Fiske and Subbarow (22). Results are expressed as micromoles of P_i released per minute per milligram of protein.

In the ^{32}P assay, ATPase activity was determined by measuring the release of ^{32}P from [γ^{-32}P]ATP (33) in a reaction mixture (0.2 ml) containing membrane protein (25 μg), 50 mM Tris-MES (morpholineethanesulfonic acid) buffer (with or without 10 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM ATP, and 0.6 μCi of a fresh preparation of [γ^{-32}P]ATP per ml). Samples were incubated at 37°C, and the reaction was stopped by the addition of 0.9 ml of a suspension containing 10% (wt/vol) activated charcoal in 50 mM KH_2PO_4 (pH 2.0) and 0.1% Triton X-100. The samples were incubated at 4°C for 10 min to allow the absorption of unhydrolyzed ATP and centrifuged for 5 min in an Eppendorf microfuge. Samples (100 μl) of the supernatant fluid were withdrawn, and radioactivity was counted. As the time course of *M. gallisepticum* ATPase activity was linear for about 10 min at pH 5.5 but deviated from linearity in less than 5 min at pH 8.5, ATPase activity was mostly assayed after incubation for up to 2 min.

Immunoblotting assay. Membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were electroblotted onto nitrocellulose paper in a solution containing 20 mM Tris hydrochloride 100 mM glycine, and 12% (vol/vol) methanol at 700 mA at 12°C. The nitrocellulose paper was washed several times in PBS solution (136 mM NaCl, 2.6 mM KCl, 16.2 mM Na_2HPO_4) containing 0.2% (vol/vol) Tween 20. It was then incubated for 90 min at room temperature with rabbit monospecific polyclonal antibodies directed against either the β-subunit of the *E. coli* F_1F_0-ATPase (20) or the yeast plasma membrane ATPase (both kindly provided by N. Nelson, Roche Institute of Molecular Biology). This incubation was followed by a further incubation with ^{125}I-labeled protein A. The papers were washed again as above, and the immune complexes were detected by autoradiography (9).

Materials. [7-^{14}C]benzoic acid and ^{125}I-labeled protein A were purchased from New England Nuclear Corp. (Boston, Mass.). [^{14}C]PEG 4000, ^3H_2O, ^{22}NaCl, [9,10-(*m*)-^{3}H]palmitate, and [γ^{-32}P]ATP (ATP tris salt) were the products of the radiochemical Centre (Amersham, UK). [^{3}H]TPP (bromine salt) was purchased from the Nuclear Research Center-Negev (Beersheba, Israel). Glycylglycine chloride, Tris, MES, MOPS, valinomycin, carbonyl cyanide *M*-chlorophenyl-hydrazone (CCCP), Na_2-ATP (vanadate free), Tris-ATP (vanadate free), sodium azide, oligomycin, sodium orthovanadate, dicyclohexylcarbodimide (DCCD), *N*-ethylmaleimide (NEM), DNase I, phenylmethylsulfonyl fluoride,

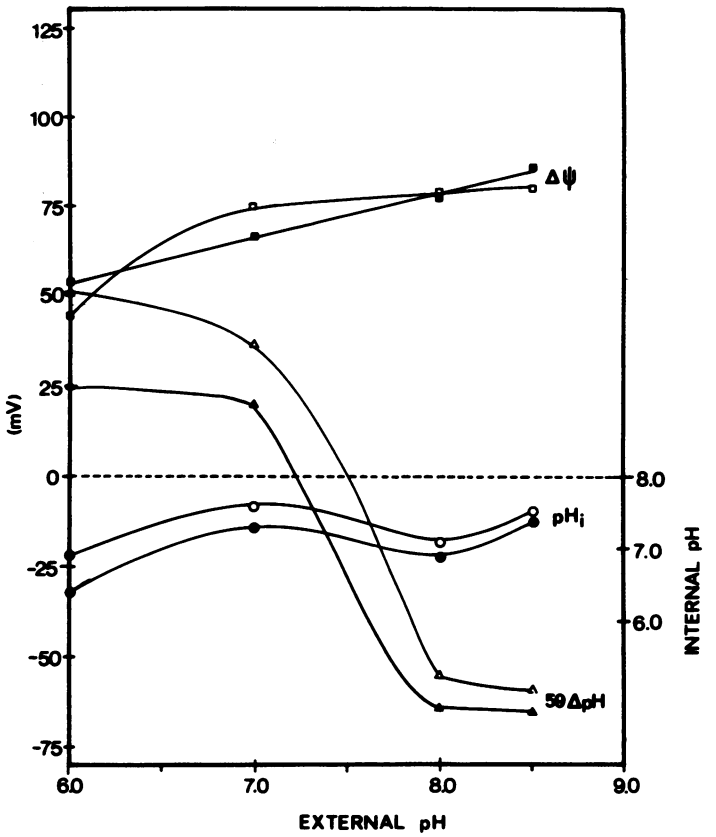

FIG. 1. Influence of pH and the uncoupler SF6847 on Δψ and ΔpH. The membrane potential (Δψ) and the chemical potential (ΔpH) were measured in *M. gallisepticum* cells suspended in 0.25 M NaCl in the absence (open symbols) or presence (solid symbols) of SF6847 (1 μM) as described in the text.

DTT, bovine serum albumin (fraction V), potassium cholate, and acridine orange were obtained from Sigma Chemical Co. (St. Louis, Mo.). Horse serum was obtained from Bio-Lab (Jerusalem, Israel). Silicone oil (550:556 grade [12:13] [vol/vol]) was obtained from Dow Corning Corp. (Midland, Mich.). Diethylstilbestrol (DES) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Zwittergent 3-12 was obtained from Calbiochem.

RESULTS

Electrochemical potentials of *M. gallisepticum*. We first sought for an active primary ion pump other than an H^+ pump by studying the electrochemical potentials of *M. gallisepticum* cells in the presence and absence of an uncoupler. The chemical potential due to the pH gradient (ΔpH) was determined by the intracellular-extracellular partitioning of either benzoic acid or methylamine, and the membrane potential (Δψ) was determined by the partitioning of TPP. Cells loaded with Na^+ and suspended in an isoosmotic NaCl solution generated a substantial proton motive force ($\Delta\tilde{\mu}H^+$), which was affected by the external pH (pH_o) over the range 6.0 to 8.5 (Fig. 1). Although the chemical potential declined to 0 mV at pH_o 7.4, the membrane potential increased from 45 mV to a maximum of approximately 75 mV. The generation of Δψ was resistant to the uncoupler SF6847 (1 μM) (Fig. 1) and CCCP (5 μM) (not shown) but inhibited by KCl (100 mM) and significantly reduced by DCCD (20 μM) (not shown). The intracellular pH (pH_i) over this pH range (6.0 to 8.5) was fairly constant. At the highest external pH tested (pH_o 8.5), the pH_i was 7.5. The chemical potential in the presence of the uncouplers SF6847 (1.0 μM) and CCCP (10 μM) was decreased by approximately 50% at pH 6.0, and at pH 8.5, where ΔpH was reversed (inside relatively acidic), it was slightly increased. The magnitude of the chemical potential at pH 8.5 was somewhat lower than that of the electrical potential (Δψ). Similar results were observed even in the presence of CCCP (5 μM) and SF6847 (1 μM).

Δψ-driven proton gradients in *M. gallisepticum*. The ability of cells to generate a Δψ by the primary extrusion of an ion other than H^+ was further studied indirectly by assaying for Δψ in the presence of an uncoupler. Generation of Δψ by the extrusion of this ion should drive H^+ uptake, acidifying the cell interior and resulting in quenching of acridine orange fluorescence. Proton uptake into sodium-loaded cells occurred only in the presence of both SF6847 (0.4 μM) and glucose (10 mM) (Fig. 2A). When the permeant cations TPP (200 μM) or KCl (25 mM) were added to collapse Δψ, fluorescence was dequenched (Fig. 2E and A, respectively). Furthermore, when KCl was added prior to the addition of glucose or SF6847, no quenching of the fluorescent signal was observed. The results suggest that both TPP (200 μM) and KCl (25 mM) were able to collapse Δψ and that 0.4 μM SF6847 at pH 8.5 was sufficient to make the membranes permeable to protons. In contrast, very little or no quenching of the fluorescent signal was observed when the assay was performed with cells preincubated for 1 h in isoosmotic choline chloride (to deplete or significantly reduce the intra-

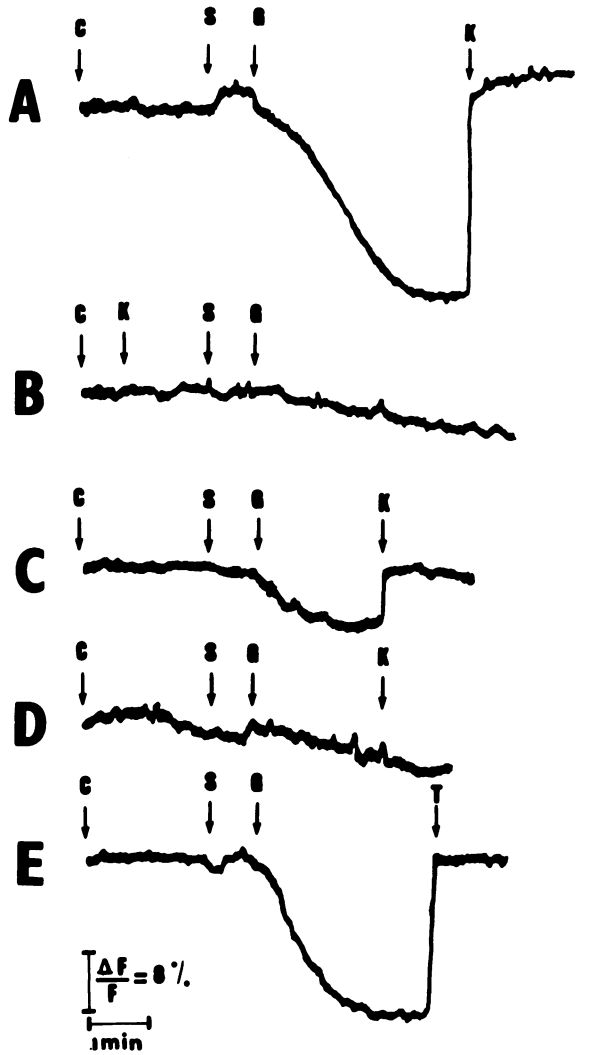

FIG. 2. Uncoupler-dependent proton uptake by *M. gallisepticum*. Proton uptake by *M. gallisepticum* cells (C) was initiated by the addition of SF6847 (S; 0.4 μM) and glucose (G; 10 mM) and monitored by the acridine orange fluorescence technique as described in the text. The ΔpH generated was collapsed by either 25 mM KCl (K) or 200 μM TPP (T). (A, B, and E) Cells loaded with sodium; (C) cell partially depleted of sodium; (D) cells loaded with potassium.

cellular concentration of sodium) before the assay and then reincubated either in choline chloride alone (Fig. 2C) or choline chloride plus 25 mM KCl (Fig. 2D), RbCl, LiCl, or CsCl (not shown) (to load the cells with potassium, rubidium, lithium, or cesium, respectively). These results are consistent with an electrogenic ion pump specific to sodium.

To test directly for an electrogenic Na^+ pump, $^{22}Na^+$ efflux from *M. gallisepticum* cells suspended in isoosmotic solutions containing both NaCl and KCl was measured (Fig. 3). Increasing the concentration of KCl (50 to 200 mM) in the efflux medium, with (not shown) or without valinomycin (10 μM), resulted in a decrease or even a collapse of Δψ, as determined by the intracellular-extracellular partitioning of TPP (not shown), and an enhancement of sodium extrusion. Treating cells with valinomycin in the absence of external potassium consistently increased Δψ (inside negative) and retarded sodium efflux.

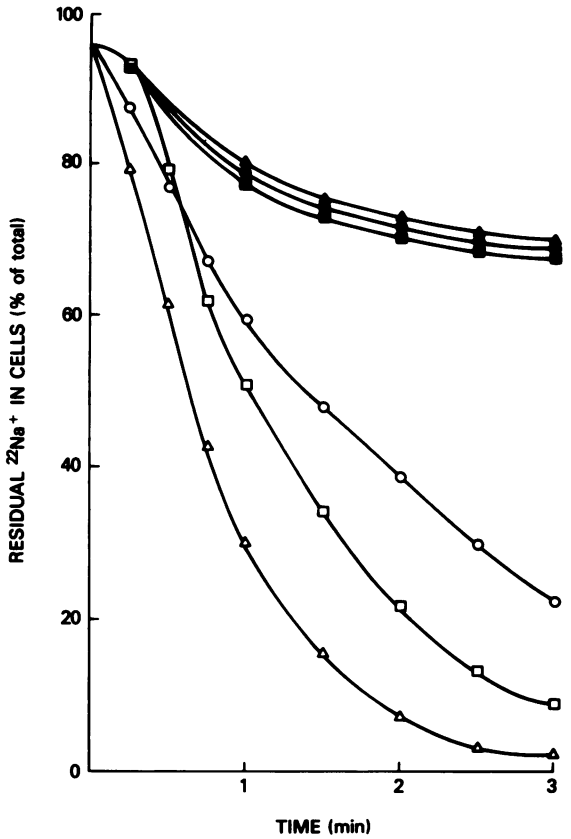

FIG. 3. Effect of the Na^+/K^+ ratio in the medium on $^{22}Na^+$ efflux. $^{22}Na^+$ efflux was monitored in 10 mM Tris-MOPS buffer (pH 7.5) with (open symbols) or without (solid symbols) glucose (10 mM) in a reaction mixture containing 200 mM NaCl (○, ●), 50 mM NaCl plus 100 mM KCl (□, ■), or 50 mM NaCl plus 200 mM KCl (△, ▲).

As a first step toward identifying the driving force for the primary sodium pump, the effect of ATPase inhibitors on sodium-pumping activity in cells was studied. Sodium pumping was monitored by the fluorometric assay used above, whereby the generation of Δψ (created from Na^+ extrusion) results in uncoupler-dependent intracellular acidification and subsequent quenching of the fluorescent signal. The generation of Δψ was inhibited by DCCD (50 μM) and DES (50 μM), but not by vanadate (100 μM) or azide (1 mM) (Fig. 4). As there was only very little dequenching with high concentrations of NEM (200 μM), which remained constant, Δψ generation was probably not inhibited by this inhibitor either.

ATPase activity in *M. gallisepticum* membranes. The ATPase activity of *M. gallisepticum* membranes at various pHs is shown in Fig. 5. No sharp pH optimum of total membrane-bound ATPase activity was observed. In the absence of sodium, ATPase activity steadily increased from pH 5.5 to about pH 7.0 to 7.5 and then rapidly decreased with increasing pHs. In the presence of sodium (10 mM), however, activity increased at the high pH range up to pH 9.0 to 9.5, at which point activity began to decrease (Fig. 5). Sodium (up to 100 mM) had no effect on activity at pH 5.5, but markedly stimulated ATPase activity at pH 8.5 (Fig. 6). The pH profile of the sodium-stimulated ATPase activity, defined as the difference between the activity in the presence of sodium and that in its absence, had a pH optimun of 9.0 to 9.5 (Fig. 5). The ATPase activity over the pH range tested

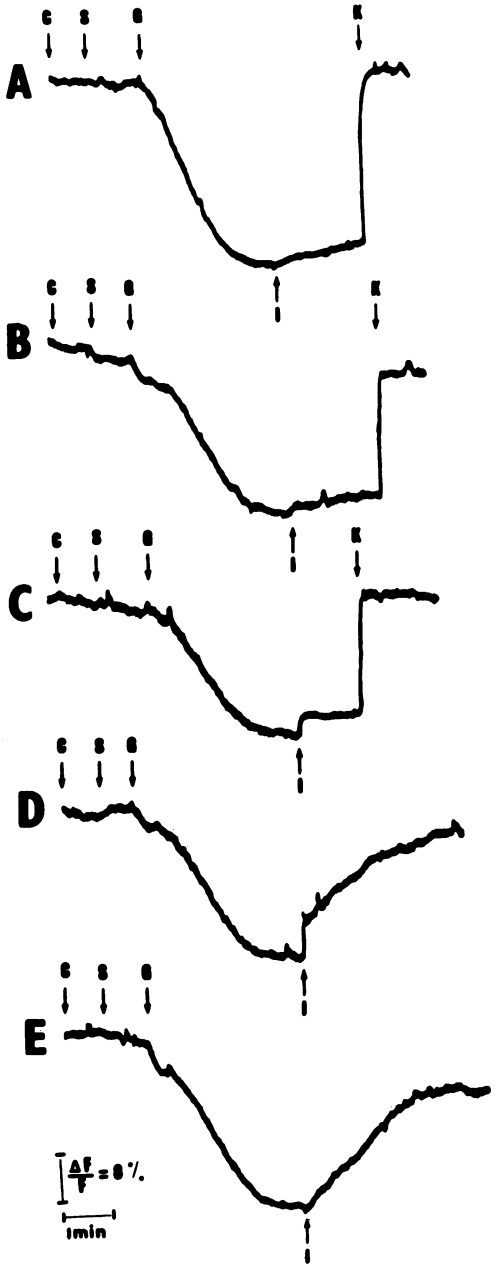

FIG. 4. Effect of inhibitors on uncoupler-dependent proton uptake by *M. gallisepticum* cells. Proton uptake was initiated in sodium-loaded cells (C) by the addition of SF6847 (S; 0.4 μM) and glucose (G; 10 mM). The effect of inhibitors (I) on the ΔpH generated was followed by the acridine orange fluorescence technique. (A) Vanadate (100 μM); (B) azide (1 mM); (C) NEM (200 μM); (D) DCCD (50 μM); (E) DES (50 μM). The ΔpH generated was finally collapsed by the addition of 25 mM KCl (K).

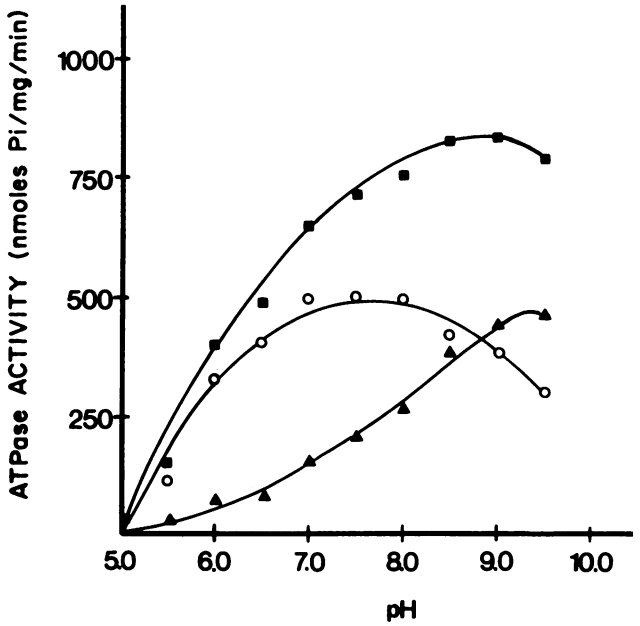

FIG. 5. Effect of pH and Na^+ on ATPase activity of *M. gallisepticum* membrane. ATPase was measured by the ^{32}P assay as described in Materials and Methods in the presence (■) or absence (○) of 10 mM Na^+. ATPase activity in the absence of Na^+ was assayed for 10 min and in the presence of Na^+ for 2 min. A differential curve obtained by subtracting ATPase activity in the absence of sodium from the activity in the presence of sodium is also shown (▲).

(5.5 to 9.5) was magnesium dependent (not shown) and was not influenced by potassium (25 mM).

It has been shown previously that the ATPase activity of either native membranes or solubilized membrane material was rapidly lost after storage at either 4 or −20°C. The addition of 25% glycerol and 1.0 mM DTT to a membrane suspension in an isoosmotic NaCl solution at pH 7.5 resulted in the retention, after 1 week of storage at 4°C, of 83% of the activity when tested at pH 8.5 in the presence of sodium (50 mM) and of 31% of the activity when tested at pH 5.5. Quick freezing of the membranes in liquid nitrogen and storage at −70°C had no significant effect on stability. Solubilizing the membranes with potassium cholate (1.0%), Triton X-100 (0.1%), or Zwittergent 3-12 (0.1%) had no significant effect on the ATPase activity of the total mixture. As with native membranes, the ATPase activity at pH 8.5 (plus 50 mM Na^+) of a stored cholate-solubilized membrane preparation was relatively stable, whereas the activity at pH 5.5 was not (Table 1).

The effect of inhibitors on the ATPase activity is shown in Table 2. Inhibition of ATPase activity was assayed at pH 7.5 in isolated membranes prepared by the sonication procedure. ATP hydrolysis was strongly inhibited by DCCD (25 μM), DES (50 to 100 μM), and quercetin (200 μM); moderately inhibited by azide (200 to 1,000 μM) and vanadate (100 μM); but not inhibited by oligomycin (100 μg/ml), ouabain (150 μg/ml), or NEM (250 μM).

Indications for two distinct ATPases. The pH profile and inhibition pattern of the total membrane-bound ATPase activity suggested the presence of two distinct ATPases in the *M. gallisepticum* membrane. Immunological cross-activity between monospecific polyclonal antiserum raised against the 52-kilodalton (kDa) β-subunit of *E. coli* F_1F_0-ATPase and a protein present in the membrane of *M. gallisepticum* was demonstrated by us before, suggesting the presence of an F_1F_0-ATPase. When membrane proteins, separated by SDS-PAGE, were blotted onto nitrocellulose and reacted with monospecific polyclonal antiserum raised against the yeast plasma membrane proton ATPase, the antiserum reacted strongly with a protein present in the *M. gallisepticum* membranes (Fig. 7C) and in *Acholeplasma laidlawii* membranes (Fig. 7A), but not in *M. capricolum*

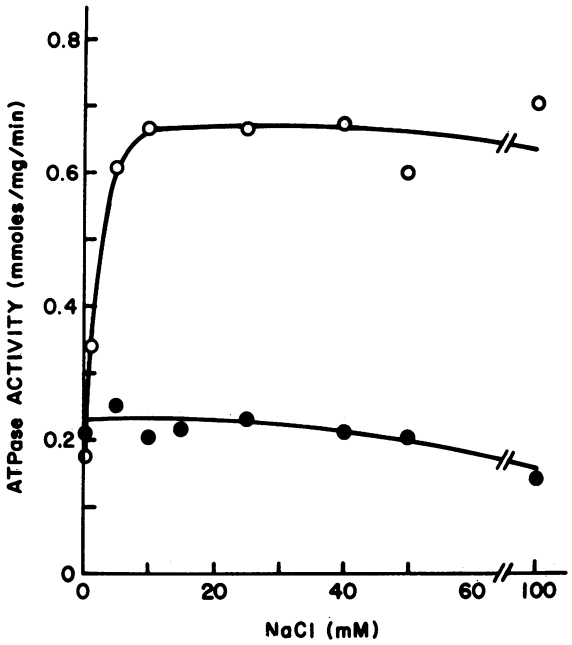

FIG. 6. Na^+ dependence of *M. gallisepticum* ATPase activity. ATPase activity was followed by *M. gallisepticum* membranes at pH 5.5 (●) and pH 8.5 (○) in the presence of increasing concentrations of NaCl. The activity was measured for 2 min by the ^{32}P assay as described in the text.

membranes (Fig. 7B). The reactive protein had an apparent molecular mass of 68 kDa. After exposure of the nitrocellulose blot to the yeast antiserum and ^{125}I-labeled protein A, the same blot was re-exposed to anti-*E. coli* β-subunit antiserum followed by the ^{125}I-labeled protein A. *M. gallisepticum* (lane C) had both a 52-kDa band (reacting with the anti-β-subunit antiserum) and a 68-kDa band (reacting with the anti-yeast ATPase antiserum), while *M. capricolum* (lane B) possessed only the 52-kDa band.

DISCUSSION

The translocation of Na^+ out of *M. gallisepticum* cells, as shown in this study by following the internal acidification of energized cells loaded with Na^+ in the presence of a proton uncoupler, is the first evidence showing primary Na^+ pumping in *M. gallisepticum*. Our observations that the ATPase inhibitors DCCD and DES can inhibit, to a large extent, the above translocation of Na^+ as well as $^{22}Na^+$ efflux and membrane-bound ATPase activity suggest that the Na^+ pump is an ATPase.

Sodium extrusion by a sodium-ATPase has been proposed for *M. mycoides* (2) and *Streptococcus faecalis* (6). These mechanisms, however, differ from sodium efflux in *M. gallisepticum* in that they require potassium for efflux and are resistant to DCCD. *M. gallisepticum* has no potassium requirement, and efflux is inhibited by DCCD. Although inhibition of the Na^+-ATPase by DCCD contradicts the notion that DCCD is a specific inhibitor of proton-translocating ATPases, it is consistent with current reports indicat-

TABLE 1. Effect of storage on the ATPase activity of solubilized *M. gallisepticum* membrane preparation[a]

Treatment of membranes	% of initial ATPase activity remaining after storage at indicated temp, determined at:			
	pH 5.5		pH 8.5	
	4°C	−70°C	4°C	−70°C
None	31	19	83	86
Triton X-100 (0.1%)	42	12	100	50
Potassium cholate (1.0%)	22	40	91	88
Zwittergent 3-12 (0.1%)	33	4	54	39

[a] Membranes were prepared from cells by the glycerol lysis procedure as described in Materials and Methods and immediately solubilized with the detergent indicated in the presence of 25% glycerol (%), 1 mM DTT, and 10 mM Tris hydrochloride (pH 7.5). The suspension was either stored at 4°C or frozen in liquid nitrogen and stored at −70°C. Specific ATPase activity was determined at either pH 5.5 or pH 8.5.

TABLE 2. Effect of inhibitors on the ATPase activity of *M. gallisepticum* membranes[a]

Inhibitor	Concn (μM)	% Inhibition of ATPase
DCCD	25	67
	100	67
DES	25	42
	100	82
Quercetin	200	73
Azide	200	37
	1,000	46
Vanadate	50	14
	100	24
Ouabain	150	0
Oligomycin	100	0
NEM		
pH 6.0	10	0
	250	12
pH 8.5	10	0
	250	15

[a] *M. gallisepticum* membranes were prepared by the sonication method. ATPase activity was determined as described in Materials and Methods at either pH 7.5 or, when treated with NEM, at pH 6.0 or 8.5.

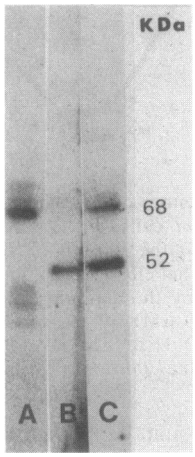

FIG. 7. Antigenic cross-reactivity of mycoplasmal proteins with both the yeast plasma membrane ATPase and the β-subunit of the *E. coli* ATPase. *M. gallisepticum* membrane proteins were separated and transferred to nitrocellulose as described in the text. The protein blot was first immunoreacted with rabbit anti-yeast plasma membrane ATPase antiserum and then with rabbit anti-*E. coli* β-subunit antiserum. (A) *A. laidlawii*; (B) *M. capricolum*; (C) *M. gallisepticum*.

ing that DCCD acts as a more general ATPase inhibitor by covalently binding to carboxylate groups (16). Furthermore, DCCD has been shown to inactivate a number of small-intestinal sodium-dependent transport systems, while not affecting sodium-independent systems (31), suggesting a role for a carboxyl group(s) in sodium transport.

It has been put forth that all ancient bacteria possess an F_1F_0-ATPase (1, 4). Consistent with this notion, the β-subunit of an F_1F_0-ATPase was immunologically identified in the membranes of *M. gallisepticum* as well as other mycoplasmas by using monospecific polyclonal antibodies against the β-subunit of the *E. coli* F_1F_0-ATPase (33). The presence of two ATPases in the *M. gallisepticum* membrane, a proton-ATPase and an Na^+-translocating ATPase, is supported by the following observations. (i) Treatment of solubilized membranes with antiserum against the β-subunit, at pH 8.3, quantitatively immunoprecipitated all of the β-subunit, while only 44% of the solubilized ATPase activity was immunoprecipitated (33). (ii) A 52-kDa membrane protein reacted with the anti-*E. coli* subunit antiserum, and a 68-kDa protein reacted with the anti-yeast plasma membrane ATPase antiserum. (iii) Two pH optima for ATPase activities were observed. (iv) ATPase activity at pH 8.5 was markedly stimulated by Na^+, whereas Na^+ had very little effect on ATPase activity at pH 5.5 (v) The ATPase activity at pH 8.5 was more stable to prolonged storage.

Membrane-bound proton pumps are generally believed to maintain $\Delta\tilde{\mu}H^+$ in bacterial cells (15). Consistently, Linker and Wilson (10–12) concluded that an electrogenic proton ATPase generates both ΔpH and Δψ in *M. gallisepticum*. If this is the case, then making the membrane permeable to protons by the addition of uncouplers should result in the collapse of Δψ. Yet no inhibition of Δψ was observed in the presence of CCCP or SF6847 over the physiological pH growth range (Fig. 1), indicating that Δψ is generated by the primary extrusion of an ion other than H^+. In view of the electrogenic nature of the proposed Na^+-ATPase, it seems that Δψ is generated by a primary Na^+ pump.

A mechanism can therefore be proposed whereby the intracellular pH of *M. gallisepticum* is regulated by Δψ, which in turn is maintained by the Na^+-ATPase. The growth of *M. gallisepticum* in the presence of CCCP (27) supports the above conclusions. The reduction of ΔpH by approximately half at pH 6.0 in the presence of SF6847 is indicative of a nonequilibrium distribution of protons at the lower pHs. This may suggest an activity of an H^+-ATPase at this pH range or that the membrane is less permeable to protons at the acidic range. A moderate permeability of the membrane to protons was shown by generating a ΔpH in cells by using the ammonium chloride dilution procedure. The permeability of *M. gallisepticum* to protons may be due to the high level of disaturated lipids found in this organism (21, 22). It was suggested that segregated domains formed by these disaturated lipids cause increased permeability of the membrane to ions (25).

Growth of *M. gallisepticum* in the presence of uncouplers suggests that a sodium electrochemical gradient ($\Delta\tilde{\mu}Na^+$) is sufficient for cell growth. The presence of a primary sodium pump and the absence of Na^+/H^+ antiporter activity further suggest that sodium is a major coupling ion for secondary transport and may drive the uptake of essential nutrients, such as amino acids and carbohydrate. Such coupling is well known in eucaryotic cells as well as in marine and halophilic bacteria (8, 19, 28, 30). Nonmarine bacteria generally use proton coupling, although some, like *E. coli*, use both (19). A more intensive study of these secondary processes would better elucidate the role of Na^+ as a coupling ion in *M. gallisepticum*.

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