Volume regulation in *Spiroplasma floricola*: evidence that Na^+ is extruded by a Na^+/H^+ antiporter

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Marked cell swelling followed by lysis was observed when *Spiroplasma floricola* cells were incubated in iso-osmotic solutions of NaCl, KCl, choline chloride or sorbitol in the absence of an energy source. In the presence of an energy source the cells did not swell suggesting that *S. floricola* relies on an energy-dependent mechanism(s) for cell volume regulation. An ammonium chloride dilution procedure was utilized to generate a pH gradient (inside acid) across the cell membrane of *S. floricola* cells. The addition of NaCl resulted in an intracellular alkalization suggesting the presence of a Na^+/H^+ exchange activity. In $^{22}\text{Na}^+$ -loaded cells, glucose-dependent $^{22}\text{Na}^+$ extrusion was observed at acidic pH in both the presence and absence of Na^+ ions. The extrusion was completely inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 μM) and partially inhibited by dicyclohexylcarbodiimide (DCCD, 100 μM) indicating that in *S. floricola*, Na^+ movement is driven by the electrochemical gradient of H^+ via a Na^+/H^+ antiporter. The specific ATPase activity of *S. floricola* membranes was at least twofold higher than that described in other mollicutes. Activity was Mg^{2+} -dependent over the pH range (6.5–8.5) tested, but was very little affected by Na^+ (up to 100 mM). DCCD (25 μM) markedly inhibited both membrane-bound and solubilized ATPase activity, whereas orthovanadate (50 μM) had only a small inhibitory effect. The properties of the enzyme are consistent with a F_0F_1 -ATPase. It is suggested that the enzyme operates in the direction of hydrolysing ATP formed by glycolysis leading to the generation of a ΔpH , which is the major driving force for the Na^+/H^+ antiport activity.

Keywords: Mollicutes, *Spiroplasma floricola*, volume regulation, Na^+/H^+ antiporter, F_0F_1 -ATPase

INTRODUCTION

Mollicutes are among the simplest autonomously replicating micro-organisms (Neimark, 1986). Their cells lack a rigid cell wall (Razin, 1981) and are bound by a single membrane; thus mollicutes are vulnerable to osmotic stress (Razin, 1981). Even in a medium isotonic with the intracellular environment, salts and water enter cells due to Gibbs–Donnan effects, causing swelling and eventually lysis of the cells (Wilson, 1954; Rottem *et al.*, 1981). Therefore, it was suggested that mollicutes must

rely on ionic pumps for one of the most basic physiological functions, the extrusion of ions and water for cell volume regulation (Linker & Wilson, 1985a; Shirvan & Rottem, 1993).

Most cells and organelles utilize antiport systems for the removal of cations from internal spaces (Wilson & Lin, 1980). In eukaryotes, the most widely used antiport systems are Na^+/H^+ ATPases, i.e. primary systems that utilize chemical energy as a primary energy source (Harold & Kakinuma, 1985). Secondary active antiport systems use the electrochemical gradients set up by a primary system, e.g. the F_0F_1 -ATPase system, present in many bacteria, which is a primary H^+ -translocating system that creates a proton gradient that drives the secondary

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DES, diethylstilbestrol.

Na^+/H^+ antiporter (Krulwich, 1983). Mollicutes have diverse natural habitats and have apparently evolved different mechanisms to offset the inward passive influx of solutes into cells. *Mycoplasma* and *Acholeplasma* species are primarily associated with vertebrates and must contend with an environment which is rich in sodium. In the plasma membrane of *Mycoplasma gallisepticum*, the presence of unusual segregated lipid domains is responsible for a high permeability to extracellular Na^+ (Rottem & Verkleij, 1982). Therefore, volume regulation in such organisms will be achieved by the pumping out of Na^+ (Shirvan & Rottem, 1993). Indeed, energy-dependent primary Na^+ -pumps have been implicated in volume regulation of *Acholeplasma* and *Mycoplasma* species (Lewis & McElhaney, 1983; Shirvan *et al.*, 1989a, b). In *M. gallisepticum*, a Na^+ -stimulated dicyclohexylcarbodiimide (DCCD)-sensitive ATPase has been described (Rottem *et al.*, 1981), and it was postulated that this enzyme acts as a primary Na^+ -pump (Shirvan *et al.*, 1989b). Secondary Na^+ antiporters have also been suggested (Linker & Wilson, 1985b; Shirvan *et al.*, 1989b; Shirvan & Rottem, 1993). Mollicutes belonging to the genus *Spiroplasma* are mostly parasites of plants and insects and, in contrast to *Mycoplasma* and *Acholeplasma* species, live in a Na^+ -poor environment. The purpose of the present study was to investigate volume regulation and Na^+ -extrusion mechanisms in *Spiroplasma floricola*, an organism isolated from flowers of *Magnolia grandiflora*.

METHODS

Organisms, growth conditions and preparation of cell suspensions. *Spiroplasma floricola* (strain BNR1) was kindly provided by R. F. Whitcomb (Beltsville, Maryland, USA). The organism was grown at 32 °C in 500 ml volumes of a modified Saglio medium (Whitcomb, 1983) inoculated with 1% (v/v) of a starter culture. *Acholeplasma laidlawii* (strain OR) was taken from our collection and was grown as previously described (Rottem *et al.*, 1981). After 18–24 h of incubation ($\text{OD}_{640} = 0.18\text{--}0.22$), the cells were harvested by centrifugation at 12000 *g* for 10 min, washed once, and resuspended in various buffered isotonic solutions. To label membrane lipids, 0.02 μCi (740 Bq) [$9,10\text{-}^3\text{H}$]palmitic acid ml^{-1} [500 μCi ml^{-1} (18.5 MBq mol^{-1})] were added to the growth medium.

Swelling measurements. Washed cell suspensions were diluted in various isotonic media to an absorbance of 0.9–1.0 at 440 nm. They were then incubated statically at 32 °C for up to 3 h and cell swelling was followed spectrophotometrically by measuring the decrease in absorbance at 440 nm.

Membrane preparations. Membranes were isolated from washed *S. floricola* cells by osmotic lysis (Razin, 1981), sonication (Rottem & Markowitz, 1979), or using diethylstilbesterol (DES). For DES-lysis, DES (final concentration 50 μM) was added to cells (2–4 mg cell protein ml^{-1}) suspended in 225 mM NaCl solution containing 25 mM Tris/HCl buffer (pH 7.5). The suspension was incubated at 32 °C for 30 min and membranes were collected by centrifugation at 34000 *g* for 30 min. The membranes were washed twice in the NaCl/Tris solution.

Acridine orange fluorescence measurements. Values of ΔpH across the cell membrane were estimated by monitoring the fluorescence quenching of acridine orange (Goldberg *et al.*,

1987; Shirvan *et al.*, 1989a) in a Perkin-Elmer spectrofluorimeter with excitation at 492 nm and emission at 530 nm. Na^+/H^+ exchange activity was tested by imposing a pH gradient (interior acid) across the cell membrane and determining the effect of Na^+ and K^+ on the gradient generated. The pH gradient was generated by the ammonium chloride dilution procedure described previously (Goldberg *et al.*, 1981; Shirvan *et al.*, 1989a). Cells were loaded with ammonium ions and diluted into ammonium-free medium. NH_4^+ -loaded cells were obtained by incubating washed cells in 0.25 M NH_4Cl containing 5 mM Tris/HCl buffer (pH 7.5) and 2.5 mM MgSO_4 for 1 h at 32 °C. The ΔpH was generated by a 125-fold dilution of the NH_4^+ -loaded cells (640 μg protein ml^{-1}) in 2.5 ml of an expression buffer containing 0.25 M choline chloride, 10 mM Tris/HCl (pH 7.5), 2.5 mM MgSO_4 and 10 μM acridine orange. Na^+/H^+ antiport activity was then tested by measuring fluorescence levels following the addition of 25 mM NaCl to the reaction mixture.

Primary Na^+ pumping activity was assayed by monitoring changes in ΔpH due to the membrane-potential-driven uptake of H^+ by cells in the presence of an uncoupler (Dimroth, 1987; Shirvan *et al.*, 1989b). Cells (75 μg protein) suspended in 2.5 ml of a solution containing 225 mM NaCl and 5 mM MgCl_2 in Tris/MES buffer (20 mM Tris adjusted with 2 M MES to various pH values) and 10 μM acridine orange were made permeable to H^+ by the addition of 0.4 μM SF6847 (kindly provided by E. Pick, The Weizmann Institute). Fluorescence quenching was initiated by the addition of an energy source (10 mM glucose) and followed at room temperature.

$^{22}\text{Na}^+$ efflux. $^{22}\text{Na}^+$ efflux measurements were performed with cells (6 mg cell protein ml^{-1}) preloaded with $^{22}\text{Na}^+$ by incubation for 1 h at 32 °C in a solution containing 25 mM NaCl, 200 mM choline chloride, 10 mM MgCl_2 and 3.0 μCi (111 kBq) $^{22}\text{Na}^+$ ml^{-1} in Tris/MES buffer (pH 7.5). The loaded cells were diluted 1:200 into a reaction mixture containing 110 ml of an isotonic solution of either NaCl or choline chloride (225 mM) in Tris/MES buffer adjusted to various pH values and containing 5 mM MgCl_2 with or without 10 mM glucose. The reaction mixtures were incubated at room temperature and, at the indicated time intervals, 10 ml portions were withdrawn and filtered through fibreglass filters (25 mm GF/C; Whatman) under negative pressure (filtration time, 3 s). The filters were washed twice with 10 ml of cold 0.25 M NaCl and the radioactivity retained on the filters was determined. Over 95% of cells were retained on the filters, as judged from measuring the retention of [^{14}C]palmitate-labelled cells.

Measurement of ATPase activity. ATPase activity was determined by measuring the release of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP (Zilberstein *et al.*, 1986) in a reaction mixture (0.2 ml) containing membrane protein (25 μg), Tris/MES buffer (pH 7.5, unless otherwise stated), 2.5 mM MgCl_2 , 2.5 mM ATP and 0.6 μCi (22.2 kBq) of a fresh preparation of [$\gamma\text{-}^{32}\text{P}$]ATP ml^{-1} . Samples were incubated at 37 °C and the reaction was stopped by the addition of 0.9 ml of suspension containing 10% (w/v) activated charcoal in 50 mM KH_2PO_4 (pH 2.0) and 0.2% Triton X-100. The samples were incubated at 4 °C for 10 min to allow the absorption of unhydrolysed 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 *S. floricola* ATPase activity was linear for about 5 min, ATPase activity was mostly assayed after incubation for up to 2 min.

Analytical methods. Protein in cell and membrane suspensions was determined by the Lowry Method. Lipids were extracted

from cell and membrane preparations by the method of Bligh & Dyer (1959), and the phospholipids were separated from the neutral lipids as previously described (Rottem, 1983). For ATP measurement, a sample of cells (0.5 mg cell protein) was placed in a test-tube containing 2 ml deionized water and boiled immediately for 2 min; ATP was then determined by the luciferase method of Cole *et al.* (1967). Intracellular water volume was determined as described previously (Rottem *et al.*, 1981). In brief, cells pre-incubated with ³H₂O water and [¹⁴C]inulin, were pipetted onto the surface of silicone oil in Microfuge tubes and centrifuged at 12800 *g* for 2 min. Under these conditions, the cells pass through the silicone oil and form a pellet at the bottom of the tube. The aqueous phase remains above the oil. Samples of the pellet and the aqueous phase were taken for radioactive analysis. ³H₂O was a measure of total pellet water, whereas [¹⁴C]inulin was a measure of intercellular space. The water space minus the inulin space was taken as the intracellular water space. Reduced adenine dinucleotide (NADH) dehydrogenase activity was determined spectrophotometrically, as previously described (Chandler *et al.*, 1989).

Materials. [¹⁴C]Polyethylene glycol, ³H₂O, [9,10-³H]palmitic acid, [¹⁴C]inulin and [γ -³²P]ATP were from Amersham. Tris, MOPS, MES, sorbitol, lysine chloride, sodium fluoride, NADH, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ATP-Tris salt, dicyclohexylcarbodiimide (DCCD), bovine serum albumin (fraction V), sodium orthovanadate and acridine orange were from Sigma. Diethylstilbestrol (DES) was from Aldrich. Zwittergent 3-12 was from Calbiochem.

RESULTS

Swelling of *S. floricola* cells

The cell volume of *A. laidlawii* cells increased from 2.25 to 3.64 μ l (mg cell protein)⁻¹ as medium osmolarity was reduced from 500 to 200 mOsm. In contrast, the corresponding change in the cell volume of *S. floricola* cells was from 3.5 to 3.88 μ l (mg cell protein)⁻¹, suggesting the presence of an intracellular structure preventing increase in cell volume. At osmolarities lower than 200 mOsm, *S. floricola* cells failed to pellet through silicone oil; thus measurements of intracellular water were not possible by this technique. The swelling of *S. floricola* cells in various isotonic solutions was investigated by following changes in optical density of a cell suspension incubated at 32 °C for several hours. Fig. 1 shows that in the absence of glucose, marked swelling followed by cell lysis was observed when *S. floricola* cells were suspended in solutions of NaCl, KCl, choline chloride or sorbitol. Similar results were obtained with lysine chloride (data not shown). The rate and extent of swelling were higher with NaCl and choline chloride than with KCl and sorbitol. The data suggest that *S. floricola* cells are permeable to a variety of ionic and nonionic low molecular mass solutes. Fig. 1 also shows that *S. floricola* cells did not swell in the presence of 10 mM glucose. Glucose could be replaced by fructose or arginine (known alternative energy sources for *S. floricola*; Whitcomb, 1983), but not by lactose, sorbitol, or the non-metabolizable analogue of glucose, α -methylglucoside (data not shown), suggesting that glucose is required for ATP generation. The intracellular level of ATP measured in cells incubated with either 10 mM glucose or arginine was 3–4 nmol ATP

(mg cell protein)⁻¹, whereas in cells incubated for 1 h in 225 mM NaCl without energy source, about 0.5 nmol ATP (mg cell protein)⁻¹ was found. The addition of glucose 2 h after swelling began neither reversed nor inhibited the swelling of *S. floricola* cells suspended in 225 mM NaCl (data not shown). Cells suspended in 225 mM NaCl without glucose swelled more rapidly at pH 8.5 (data not shown) than at pH 7.5 (Fig. 1). At pH 6.5, cell swelling was very low or undetectable.

Effect of inhibitors on swelling

The effect of various inhibitors on the swelling of *S. floricola* cells suspended in 225 mM NaCl with 10 mM glucose is shown in Fig. 2. The H⁺ ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 μ M) which collapses the chemical components of the protonmotive force, as well as dicyclohexylcarbodiimide (DCCD, 100 μ M), an inhibitor of the membrane-bound ATPase (Pederson & Carafoli, 1987), and NaF (25 mM, data not shown), did not induce cell swelling. Swelling was induced, however, by diethylstilbestrol (DES). DES (50 μ M) did not affect the rate or extent of ATP biosynthesis in cells incubated for up to 12 min in 225 mM NaCl at 32 °C, and only partially (10–25%) inhibited ATPase activity. Therefore, it seems that DES induces cell swelling and lysis even in the presence of high intracellular ATP levels. Substitution of NaCl by isotonic solutions of KCl, choline chloride or sorbitol failed to prevent swelling.

Membrane isolation by DES-induced lysis

The high sensitivity of *S. floricola* cells to DES led us to develop a gentle method for membrane preparation. To isolate the membranes, *S. floricola* cells (2–4 mg cell protein ml⁻¹) were suspended in 0.25 mM NaCl solution containing 25 mM Tris buffer (pH 7.5). DES was then added to a final concentration of 50 μ M and the suspension incubated at 32 °C for 30 min. Membranes were then collected by centrifugation, washed and resuspended as described in Methods. Some properties of the DES-membranes are presented in Table 1 and are compared to membranes obtained by ultrasonic treatment or osmotic lysis. The DES-induced membranes contained 50% of the total cell protein and over 95% of the radioactive phospholipids. Membranes prepared by ultrasonic treatment contained only 67% of the radioactive phospholipids, suggesting that *S. floricola* membranes are fragile and tend, upon ultrasonication, to disintegrate into fragments not sedimentable at 34000 *g* (10 min). The membranes obtained by DES-induced cell lysis had a comparatively low NADH dehydrogenase activity. Approximately 95% of the activity of whole cells was recovered in the supernatant fluid, showing good separation of this cytoplasmic enzyme from membranes. However, DES-prepared membranes retained membrane-bound ATPase activity; this was somewhat lower than expected, apparently because of partial inhibition (about 20%) induced by DES.

Fig. 1

Fig. 2

Fig. 1. Swelling of *S. floricola* cells. Cell swelling was measured as decrease in optical density (OD_{440}) of cells suspended (initial $OD_{440} = 0.8-1.0$) at 32°C in 25 mM Tris/HCl buffer (pH 7.5) containing ($\circ$, $\bullet$) 225 mM NaCl; ($\square$, $\blacksquare$) 225 mM KCl; ($\triangle$, $\blacktriangle$) 225 mM choline chloride; ($\diamond$, $\blacklozenge$) 450 mM sorbitol. Open symbols, with 10 mM glucose; filled symbols, without glucose.

Fig. 2. Effect of inhibitors on the swelling of *S. floricola* cells. Cell swelling was monitored as described in the legend to Figure 1. Cells were suspended in 225 mM NaCl solution containing 25 mM Tris/HCl buffer (pH 7.5) and 10 mM glucose either without inhibitor ($\circ$), or with: 100 μM DCCD ($\bullet$); 10 μM CCCP ($\blacktriangle$); 10 μM DES ($\square$); 20 μM DES ($\blacksquare$); or 50 μM DES ($\blacksquare$).

Table 1. Properties of *S. floricola* membrane preparations

Cells were grown in a medium containing 4% (v/v) horse serum and $0.01 \mu\text{Ci}$ (370 Bq) [^3H]palmitate ml^{-1} .

Preparation	Yield (%)		Phospholipid to protein ratio [c.p.m. (mg protein^{-1})]	NADH dehydrogenase activity*	ATPase activity†
	Protein	Phospholipids			
Intact cells	100	100	45 600	6.1	500
Membranes obtained by:					
Osmotic lysis	69	95	61 700	1.9	820
Ultrasonic treatment	36	67	74 400	0.7	720
DES treatment	50	96	82 700	0.3	660

* NADH dehydrogenase activity was expressed as the change in absorbance at $340 \text{ nm min}^{-1} (\text{mg protein}^{-1})^{-1}$.

† ATPase activity was measured at pH 7.5 and is expressed as $\text{nmol ATP hydrolysed min}^{-1} (\text{mg protein}^{-1})^{-1}$.

Effect of transmembrane pH gradient on Na^+ uptake

A transmembrane ΔpH was generated in intact *S. floricola* cells by equilibrating cells with NH_4Cl and diluting them into 250 mM choline chloride solution buffered at pH 7.5.

The dissociation of NH_4^+ to NH_3 and H^+ , and the diffusion of NH_3 out of the cells caused acidification of the cell interior that was monitored from the quenching of acridine orange. The acidity was maintained as long as Na^+ was absent from the medium. Addition of

Fig. 3

Fig. 4

Fig. 3. Effect of sodium and glucose on $^{22}\text{Na}^+$ efflux. Cells equilibrated with 25 mM $^{22}\text{Na}^+$ were transferred to 225 mM NaCl (□, ■) or choline chloride (○, ●) in Tris/MES buffer (pH 6.5) containing 5 mM MgCl_2 with (open symbols) or without (filled symbols) 10 mM glucose.

Fig. 4. Effect of inhibitors on $^{22}\text{Na}^+$ efflux. $^{22}\text{Na}^+$ -loaded cells were incubated in 225 mM NaCl, 5 mM MgCl_2 and 10 mM glucose in Tris/MES buffer (pH 6.5) without inhibitor or with CCCP (△, 10 μM); DCCD (□, 100 μM) or DES (○, 50 μM).

25 mM NaCl resulted in alkalization of the cell interior (data not shown). This observation suggests that under the experimental conditions described (low intracellular pH, high NH_4^+ concentrations), *S. floricola* cells exhibit an Na^+ -dependent recovery mechanism for internal pH, apparently due to Na^+/H^+ exchange activity.

$^{22}\text{Na}^+$ efflux from *S. floricola* cells

To test directly Na^+ extrusion from *S. floricola* cells, $^{22}\text{Na}^+$ efflux from *S. floricola* cells suspended in isotonic solutions of NaCl or choline chloride was measured. Fig. 3 shows the kinetics of $^{22}\text{Na}^+$ efflux. In the presence or absence of external Na^+ (250 mM NaCl), $^{22}\text{Na}^+$ efflux was glucose dependent. The efflux of $^{22}\text{Na}^+$ from energized cells was rapid and approximately linear with time for 6 min; up to 80% of $^{22}\text{Na}^+$ was released. In the absence of glucose, we were unable to detect significant $^{22}\text{Na}^+$ efflux, even from cells incubated in a sodium-free medium where efflux would be downhill.

Fig. 4 shows the effect of inhibitors on glucose-dependent $^{22}\text{Na}^+$ efflux from cells suspended in 225 mM NaCl at pH 6.5. The uncoupler CCCP (10 μM) completely inhibited $^{22}\text{Na}^+$ efflux. Similar inhibition was obtained with the uncoupler SF6847 (data not shown), consistent with the notion that Na^+ movements across the membrane are driven by ΔpH . The ATPase inhibitor DCCD partially

inhibited $^{22}\text{Na}^+$ efflux, whereas DES had little effect. At more alkaline pH (pH > 8.0), only a slight inhibition was observed with CCCP, whereas the effect of DCCD was similar to that at pH 6.5.

The ability of *S. floricola* cells to extrude Na^+ by a primary process was investigated by following the acidification of the cell interior in the presence of the uncoupler SF6847 (0.4 μM). It has been previously shown that generation of $\Delta\psi$ by extrusion of Na^+ should drive H^+ uptake, acidifying the cell interior and resulting in quenching of acridine orange fluorescence (Dimroth, 1987). H^+ uptake in the presence of SF6847 was evident with Na^+ -loaded *S. floricola* cells (incubated with NaCl for 1.5–3 h), but not with unloaded cells (Fig. 5a). H^+ uptake was also dependent upon the presence of an energy source (10 mM of either glucose or arginine) and was marked at alkaline pH (Fig. 5b). When KCl (25 mM) was added, fluorescence was dequenched, showing the collapse of $\Delta\psi$. These results may suggest an electrogenic ion pump specific for Na^+ that operates at the more alkaline pH range.

ATPase activity of *S. floricola* membranes

The ATPase activity of washed membranes of *S. floricola* was measured by determining the release of ^{32}P from [γ - ^{32}P]ATP. Activity was at least twofold higher than those described in other mollicutes (Shirvan & Rottem, 1993)

Fig. 5. H^+ uptake in the presence of uncoupler. Cells were preloaded with Na^+ by incubation in 225 mM NaCl and 5 mM $MgCl_2$ in Tris/MES buffer (pH 7.5) for various periods of time. H^+ uptake was monitored using an acridine orange fluorescence technique as described in the text in the presence of the uncoupler SF6847 (0.4 μ M) and glucose (10 mM). The transmembrane Δ pH generated was collapsed by 25 mM KCl. (a) Cells preloaded for 0, 1.5 or 3.0 h. H^+ uptake measured at pH 8.0. (b) Cells preloaded for 3 h. Proton uptake measured at various pH values. %F = percentage fluorescence.

Table 2. Effect of Na^+ on the ATPase activity of *S. floricola* membranes

NaCl (mM)	ATPase activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	pH 6.5	pH 8.5
0	692	818
1	765	942
5	830	1006
10	770	860
50	880	872

and relatively independent of pH in the range 6.5–8.5 (Tables 1 and 2). The enzyme had an obligatory requirement for Mg^{2+} that was best met at a Mg^{2+} to ATP molar ratio of 1. Since Na^+ -translocating ATPases show a marked Na^+ stimulation (Pederson & Carafoli, 1987), it was important to study the effect of Na^+ on the ATPase activity of *S. floricola*. Table 2 shows that NaCl concentrations of up to 100 mM had very little effect on activity measured at either pH 6.5 or 8.5. The relatively weak stimulation observed was also obtained with KCl and is consistent with a non-specific salt effect (Linker & Wilson, 1985c).

Table 3 shows the effect on ATPase activity of DCCD (F-type ATPase inhibitor; Futai & Kanazawa, 1983) and orthovanadate, (P-type ATPase inhibitor; Pederson &

Table 3. Effect of inhibitors on membrane-bound and solubilized ATPase activity of *S. floricola*

Membranes were isolated by ultrasonic treatment of washed *S. floricola* cells. Solubilized membranes (0.1 mg protein ml⁻¹) were prepared with 0.1 % (w/v) Zwittergent 3-12 in Tris/MES buffer (pH 7.5).

Preparation	Inhibitor	ATPase activity*
Intact membranes	None	746
	Vanadate (50 μ M)	552
	DCCD (25 μ M)	238
Solubilized membranes	None	1268
	Vanadate (50 μ M)	1096
	DCCD (25 μ M)	313

* nmol ATP hydrolysed min⁻¹ (mg membrane protein)⁻¹.

Carafoli, 1987). In order to ensure that inhibitors had complete access to the enzyme, their effects were determined on intact membranes and membrane preparations solubilized with 0.1 % *N*-dodecyl-*N,N*-dimethyl-3-ammonium-1-propane sulphonate (Zwittergent 3-12) as described by Linker & Wilson (1985c). ATPase activity in the presence of 0.1 % Zwittergent 3-12 was almost twice that recorded in its absence, and was inhibited by DCCD, but was little affected by orthovanadate. The inhibitory effect of DCCD was similar in both native and detergent-solubilized membranes but was markedly lower than that

previously reported for the *M. gallisepticum* ATPase (Shirvan *et al.*, 1989b).

DISCUSSION

Cell swelling results from the flow of water from the external medium to the intracellular membrane-bound compartment. Two factors significantly affect the entry of water: one is the intracellular colloid osmotic pressure and the other is the number of osmotically active components in the cell. A colloid osmotic pressure results from the presence of nondiffusible macromolecules within the cell (Wilson & Lin, 1950). In addition, as most of these macromolecules are polyanionic, they act to attract smaller permeable cations into the cell. This is referred to as the Gibbs–Donnan potential (Wilson, 1954). Most prokaryotes protect themselves from osmotic lysis by the presence of a rigid cell wall. Animal cells depend on active extrusion mechanisms for ions (Harold & Kakinuma, 1985). Na⁺ extrusion mechanisms have been also described in *M. gallisepticum* (Shirvan *et al.*, 1989b), *M. mycoides* subsp. *capri* (Benyousef *et al.*, 1982) and *A. laidlawii* (Lewis & McElhaney, 1983).

Our results show that an energy source is required to prevent the swelling of *S. floricola* cells in isotonic solutions of choline chloride, KCl or sorbitol, as well as in NaCl. This suggests that in *S. floricola*, energy is required mainly to maintain membrane integrity rather than for extrusion of Na⁺ from cells. Further support for this notion was obtained by showing that energized *S. floricola* cells suspended in NaCl did not swell in the presence of the uncoupler CCCP, although CCCP inhibits the Na⁺/H⁺ antiporter (see Fig. 4). Questions concerning the structural integrity of spiroplasma membranes have been raised previously (Williamson, 1989). In several spiroplasmas, intracellular filaments forming a cytoskeleton-like structure have been demonstrated, and it has been suggested that these filaments maintain membrane integrity and cell shape (Williamson, 1989). It is possible that in the absence of an energy source, the cytoskeleton-like structure will disintegrate affecting the permeability properties of the cell membrane and allowing the rapid inward diffusion of small solutes.

The addition of DES to cells resulted in rapid cell swelling despite maintenance of high intracellular ATP levels. The high sensitivity of *S. floricola* cells to DES enabled development of a method for obtaining purified membrane preparations. Its efficiency in terms of membrane yield is about 96%, as judged by the percentage of radioactive phospholipids recovered in membrane fractions. In addition, the membranes obtained were not appreciably contaminated by unlysed cells or the cytoplasmic enzyme NADH dehydrogenase. The DES-method for isolating membranes is potentially important; lysis by DES is much more gentle than subjecting cells to ultrasonic irradiation and may avoid the loss of loosely bound membrane proteins reported for osmotic-shock methods (Razin, 1981).

DES has been previously utilized for preparing membranes of *M. pneumoniae* (Chandler *et al.*, 1989). It was

suggested that, like DCCD, DES is an ATPase inhibitor (McEnery & Pedersen, 1986) and the ability to induce lysis of *M. pneumoniae* cells resulted from the inhibition of an ATPase-dependent Na⁺-extrusion mechanism (Chandler *et al.*, 1989). In *S. floricola* the inability of DCCD to induce cell lysis and the low level of ATPase inhibition caused by DES (see Table 1) suggest that this explanation is unlikely. The intracellular location of the lipophilic DES in cells has been the subject of a number of studies which suggest that DES has a low capacity for penetrating the cell membrane and thus is associated with hydrophobic membrane proteins (Martinez-Azorin *et al.*, 1992). It is, therefore, possible that in *S. floricola* DES interacts with an integral membrane protein inducing conformational transitions that affect either membrane integrity and/or the binding and dissociation of the intracellular cytoskeleton-like structure to the membrane. These effects may, in turn, leave the Gibbs–Donnan forces unopposed, leading to cell swelling and lysis.

The first indication that *S. floricola* cells may extrude Na⁺ by a combination of an H⁺-translocating ATPase and a Na⁺/H⁺ antiporter was obtained by showing a Na⁺/H⁺ exchange in cells after imposition of a pH gradient (acidic inside) by the ammonium chloride dilution procedure. We have also shown a similar Na⁺/H⁺ exchange in sealed membrane vesicles (I. Shirazi & M. H. Shirvan, unpublished data). In bacteria, two membrane-associated processes are known to involve inward H⁺ translocation. These processes are the electrogenic Na⁺/H⁺ antiport (Kulwich, 1983; Harold & Kakinuma, 1985; Padan & Schuldiner, 1986) and oxidative phosphorylation, which utilizes the activity of F₀F₁-ATPase synthase (Futai & Kanazawa, 1983). As spiroplasmas lack cytochromes and oxidative phosphorylation processes (Pollack, 1992), it is likely that the acidification of the cells that occurs selectively after the addition of Na⁺ is due to an Na⁺/H⁺ antiport activity. Further support for an active Na⁺/H⁺ antiport activity in *S. floricola* cells is based on the following evidence: (1) ²²Na⁺ efflux was completely inhibited by CCCP in the acidic pH range; (2) the growth of *S. floricola* cells in high NaCl medium was partially inhibited by CCCP (I. Shirazi, unpublished data).

In *S. floricola*, the transmembrane protonmotive potential (Δp , –123 V) was constant over a wide range of external pH values (Schummer & Schiefer, 1987) and was higher than that of previously studied mollicutes. Shirvan & Rottem (1993) demonstrated that the Δp component of Δp is generated by a membrane-bound, electrogenic, H⁺-translocating ATPase which operates in the direction of hydrolysis of ATP formed by glycolysis and, thus, leads to H⁺-extrusion (Schummer & Schiefer, 1987). Our observations in this paper suggest that Δp is the major driving force for Na⁺ extrusion from *S. floricola* cells at acidic pH.

All bacteria are believed to possess a F₀F₁-ATPase (Futai & Kanazawa, 1983). Consistent with this notion, the β -subunit of an F₀F₁-ATPase has been identified in the cell membrane of representative *Mycoplasma*, *Acholeplasma* and *Spiroplasma* species, including *S. floricola*, by utilizing monospecific polyclonal antibodies against the β -subunit

of the *E. coli* F_0F_1 -ATPase (Rottem *et al.*, 1987). Evidence for the presence of two distinct membrane ATPases in *M. gallisepticum* has recently been presented (Shirvan *et al.*, 1989b). It was suggested that one of the enzymes is an H^+ -translocating F_0F_1 -ATPase, whereas the second enzyme is a Na^+ -stimulated ATPase that may act in Na^+ translocation (Shirvan *et al.*, 1989b; Rasmussen *et al.*, 1992). Similarly, in *Spiroplasma citri*, the finding that only part of the membrane-bound ATPase activity was sensitive to vanadate suggests the presence of two ATPases (Simoneau & Labarère, 1991). The participation of membrane-bound ATPase in Na^+ -extrusion from *Mycoplasma* and *Acholeplasma* cells has been demonstrated previously (Shirvan *et al.*, 1989a; Benyousef *et al.*, 1982). ATPase may act by two alternative roles. The first involves an H^+ -translocating ATPase which generates a protonmotive force energizing a Na^+/H^+ exchange mechanism (Pederson & Carafoli, 1987). The second mechanism involves a primary ATP-driven Na^+ -transport system (Shirvan *et al.*, 1989b). Our observations in this paper suggest that the major ATPase activity of *S. floricola* membranes is H^+ -translocating, since activity was neither stimulated by Na^+ nor sensitive to vanadate. The ATPase was active over a wide pH range and may regulate the intracellular pH. The presence of Na^+/H^+ antiporter activity suggests that *S. floricola* extrudes Na^+ by a combination of the H^+ -translocating ATPase and the Na^+/H^+ antiporter as suggested in other systems (Wilson & Lin, 1980; Krulwich, 1983; Rosen, 1986). Nevertheless, at alkaline pH (pH 8.0–8.5), Na^+ movement was only little affected by collapsing ΔpH , and H^+ uncouplers caused internal acidification of energized cells loaded with Na^+ . Thus, we cannot exclude the possibility that an outward-directed primary Na^+ pump is active at the alkaline pH range.

ACKNOWLEDGEMENTS

We would like to thank A. Katzenell for technical assistance. This work was supported by the Israel–United States Binational Agriculture Research and Development Fund (grant no. 1902-90R).

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- Received 3 September 1993; revised 7 March 1994; accepted 15 March 1994.