Cation transport mechanisms in *Mycoplasma mycoides* var. Capri cells

The nature of the link between K^+ and Na^+ transport

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We have studied the links between the mechanisms of Na^+ , K^+ and H^+ movements in glycolysing *Mycoplasma mycoides* var. Capri cells. In the light of the results reported in the preceding paper [Benyoucef, Rigaud & Leblanc (1982) *Biochem. J.* **208**, 529–538], we investigated certain properties of the membrane-bound ATPase of *Mycoplasma* cells, with special reference to its ionic requirements and sensitivity to specific inhibitors. Our findings show, first, that, although Na^+ stimulated ATPase activity, K^+ did not affect it, and, secondly, that *NN'*-dicyclocarbodi-imide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) were potent inhibitors of the basal ATPase activity, which was unaffected by vanadate and ouabain. We also investigated the movements of Na^+ and H^+ under the experimental conditions applied to the study of the K^+ uptake reported in the preceding paper, and found that when ' Na^+ -loaded cells' previously equilibrated with $^{22}Na^+$ were diluted in a sodium-free medium, addition of glucose induced a rapid efflux of $^{22}Na^+$. This energy-dependent efflux was independent of the presence of KCl in the medium. Studies of the changes in internal pH by 9-aminoacridine fluorescence or [^{14}C]methylamine distribution indicated that the movement of Na^+ was coupled to that of protons moving in the opposite direction, a finding that supports the presence of an Na^+/H^+ antiport. When Na^+ -loaded cells are diluted in an Na^+ -rich medium the Na^+/H^+ antiport is still active, but cannot decrease the intracellular Na^+ concentration. Under such conditions, net $^{22}Na^+$ extrusion is specifically dependent on the presence of K^+ in the medium. The present results and those derived from the study of K^+ accumulation (the preceding paper) can be rationalized by assuming that *Mycoplasma mycoides* var. Capri cells contain two transport systems for Na^+ extrusion: an Na^+/H^+ antiport and an ATP-consuming Na^+/K^+ -exchange system.

The observations reported in the preceding paper (Benyoucef *et al.*, 1982) indicate that *Mycoplasma mycoides* var. Capri cells actively accumulate K^+ in

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Abbreviations used: ΔpH , transmembrane pH gradient; $\Delta\psi_m$, electrical potential; $\Delta\mu_{H^+}$, electrochemical proton gradient; $\Delta\mu_{K^+}$, $\Delta\mu_{Rb^+}$, $\Delta\mu_{TPMP^+}$, chemical potential difference for K^+ , Rb^+ and $TPMP^+$ respectively; Mops, 4-morpholinepropanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodi-imide; NBD, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

such way that $\Delta\mu_{K^+} > \Delta\psi_m$. Further, this active K^+ accumulation is driven by the transmembrane electrical potential and is specifically dependent on the presence of Na^+ in the medium. Finally, the Na^+ -dependent K^+ transport system concerned is a system consuming ATP.

The aim of the present study was to elucidate the precise mechanism by which Na^+ stimulates this K^+ accumulation, and more generally, the mechanism of cation regulation in *Mycoplasma mycoides* var. Capri.

The fact that Na^+ stimulates K^+ transport into the cytoplasm suggests that a link exists between the movements of the two cation species. In principle,

there are two mechanisms for coupling ion movements (Lanyi, 1979): (i) the transport component in the membrane is shared, which makes coupling obligatory, and (ii) the translocation of each species is catalysed by physically separated carriers in the membrane, thus leading to indirect coupling. When these two possibilities are considered in the light of the fact that ATP is consumed during Na^+ -dependent K^+ accumulation, catalysis of Na^+ and K^+ fluxes by an $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase similar to that present in animal cells (Glynn & Karlish, 1975; Levitt, 1980) seems the more attractive hypothesis. In the present paper, we examine this possibility by studying Na^+ and K^+ requirements and the effects of certain inhibitors on the ATPase activity present in the membrane preparations of *Mycoplasma* cells.

We then analysed the properties of the cellular Na^+ extrusion mechanism in the presence or absence of a transmembrane Na^+ gradient, paying special attention to the effects of K^+ on the Na^+ efflux.

Furthermore, since many reports have suggested that Na^+ and K^+ movements in bacteria are linked to proton circulation (for reviews, see Harold & Altendorf, 1974, 1977; Lanyi, 1979) we determined the contribution of H^+ movements to the mechanisms of Na^+ and K^+ regulation.

Our observations demonstrate that, as reported for many organisms (Harold & Papineau, 1972; West & Mitchell, 1974; Lanyi & MacDonald, 1976; Lanyi, 1979; Beck & Rosen, 1979; Mandel *et al.*, 1980), an Na^+/H^+ antiport exists in *Mycoplasma* cells. Our experiments also suggest that besides this Na^+/H^+ antiport system, *Mycoplasma mycoides* var. Capri cells may possess a specific K^+ transport system whose function allows an Na^+ extrusion concomitant with the K^+ uptake. These results and those presented in the preceding paper (Benyoucef *et al.*, 1982) are discussed in terms of the different models proposed in the literature for cationic regulation in other micro-organisms.

Materials and methods

Organism and growth conditions

Mycoplasma mycoides var. Capri (PG₃ strain) was grown and cells were prepared as described in the preceding paper (Benyoucef *et al.*, 1982).

$^{22}\text{Na}^+$ loading and transport assays

' Na^+ -loaded cells' were obtained as described previously (Benyoucef *et al.*, 1982) and contain about 100 mM- Na^+ and less than 5 mM- K^+ . These cells were incubated in a buffer containing 100 mM-sodium phosphate, 40 mM- NaCl , 2 mM- MgCl_2 , pH 7.2, and $^{22}\text{NaCl}$ (30 mCi/ml).

When the Na^+ efflux was studied against a sodium concentration gradient after 15 min in-

cubation at 37°C, cells (2 mg of protein/ml) were removed at intervals and filtered as described in the preceding paper (Benyoucef *et al.*, 1982). When the Na^+ efflux was studied in the absence of external Na^+ , $^{22}\text{Na}^+$ -equilibrated cells (30 mg/ml) were diluted 200–300-fold (to approx. 0.1 mg of protein/ml) in an unlabelled buffer containing 50 mM-Mops, 17 mM- MgO , 120 mM-choline chloride, 2 mM- MgCl_2 and 2 mM- H_3PO_4 , pH 7.2.

Fluorescence measurements

Fluorescence was measured with an MPF 44A Perkin-Elmer spectrofluorimeter as described previously (Benyoucef *et al.*, 1981b). The fluorescence of 9-aminoacridine was measured at 455 nm (λ excitation, 400 nm) and the fluorescence of Mero-cyanine 540 at 581 nm (λ excitation, 536 nm).

Determination of pH gradient

The pH gradient across the membrane was determined from the accumulation of [^{14}C]methylamine or [^3H]acetate by filtration and flow-dialysis experiments (Benyoucef *et al.*, 1981a).

Protein determination

Proteins were measured as described by Lowry *et al.* (1951) with bovine serum albumin as standard.

Determination of ATPase activity

Membranes were isolated from cells by osmotic lysis and differential centrifugation as previously described (Rigaud & Leblanc, 1981) and resuspended in a 1:20 dilution of buffer devoid of NaCl and containing 50 mM-Tris, 20 mM- β -mercaptoethanol and 160 mM-choline chloride, pH 7.0.

In our standard assay, ATPase activity was measured at 37°C in a final volume of 2.5 ml containing 50 mM-Tris, 5 mM- MgCl_2 , 50 mM- NaCl and 1 mM [γ - ^{32}P]ATP.

The reaction was started by the addition of membrane suspension (50 $\mu\text{g}/\text{ml}$). At fixed intervals, 250 μl samples were withdrawn and rapidly mixed in 300 μl of a solution containing 25% activated charcoal in 5% trichloroacetic acid (Chen *et al.*, 1956). After centrifugation at 10000 g for 2 min, 100 μl of the supernatant was collected and transferred into vials containing Instagel.

Hydrolysis rates are expressed as nmol of P_i released/min per mg of membrane protein.

When ATPase activity was measured in the absence of Na^+ , the Na^+ concentration in the assay medium was less than 30 μM .

Materials

[^3H]acetate (sp.radioactivity 20 Ci/mmol), [^{14}C]methylamine (sp.radioactivity 38.4 Ci/mmol) and $^{22}\text{NaCl}$ (1 Ci/g) were purchased from C.E.N. Saclay, Gif-Sur-Yvette, France. [γ - ^{32}P]ATP was purchased from Amersham International.

Results

ATPase activity in *Mycoplasma mycoides* var. *Capri* membranes

The observations that loss (or gain) of internal K^+ is largely compensated by a gain (or loss) of Na^+ (Leblanc & Le Grimellec, 1979a) and that cellular ATP is consumed during Na^+ -stimulated K^+ reaccumulation (Benyoucef *et al.*, 1982) make the idea that *Mycoplasma* membranes possess an $(Na^+ + K^+)$ -dependent ATPase of the type found in animal cell membranes an attractive hypothesis.

We therefore carried out detailed characterization of the ATPase activity in *Mycoplasma* membranes, with particular emphasis on its dependence on univalent cation requirements and on the effects of inhibitors. As shown in Table 1, ATPase activity at pH 7 is stimulated up to 2-fold by 100 mM-NaCl. Nevertheless this stimulation requires a fairly high concentration of Na^+ (apparent K_m around 30 mM; results not shown). The other cations tested failed to stimulate ATPase activity and, in particular, no stimulation by K^+ was seen at concentrations of up to 100 mM either in the absence of Na^+ or in the presence of 100 mM-NaCl.

Table 2 shows the effects of some inhibitors on ATPase activity. Ouabain and vanadate, which are potent inhibitors of the $(Na^+ + K^+)$ -dependent ATPase present in membranes of animal cells (Glynn, 1964; Simons, 1979) did not significantly affect the ATPase activity of mycoplasma membranes either in the absence or presence of Na^+ even at the high concentration of 0.5 mM. On the other hand, DCCD and NBD, which are specific inhibitors of Mg^{2+} -dependent ATPase in microorganisms (Simoni & Postma, 1975; Haddock & Jones, 1977) considerably inhibited *Mycoplasma* ATPase activity as shown by Leblanc & Le Grimellec (1979b). The inhibition by DCCD or NBD was independent of the presence of NaCl in the assay medium, indicating that the Na^+ -stimulated part of the ATPase activity was also inhibited by this dicyclodi-imide derivative.

All these results indicate that the ATPase activity measured in *Mycoplasma mycoides* var. *Capri* membranes represents a single enzyme species, i.e. an Mg^{2+} -dependent ATPase activated by high concentrations of Na^+ but not by K^+ . Comparable results have been reported in another *Mycoplasma* strain, *Acholeplasma laidlawii*, by Jinks *et al.* (1978), although the specific stimulation by Na^+ was observed at very low concentrations (a 3-fold increase in ATPase activity at 500 μ M). It is important to note that the lack of K^+ effect on enzyme activity is clearly inconsistent with the existence of an ATPase catalysing the exchange of Na^+ and K^+ , of the type found in eukaryotic cell membranes.

Table 1. Effects of univalent cations on ATPase activity of *Mycoplasma mycoides* var. *Capri* membranes

The basic assay system contained 1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 50 mM-Tris, 5 mM- MgCl_2 and 100 mM-choline chloride, pH 7.0. The tested cations replaced choline chloride at the concentration indicated. Reaction was started by adding membrane suspension (50 μ g/ml).

Cation	Specific activity (nmol of P_i /min per mg of protein)
None	445
Na^+ (100 mM)	900
Na^+ (50 mM)	620
K^+ (100 mM)	420
K^+ (50 mM)	430
Na^+ (50 mM) + K^+ (50 mM)	630
Li^+ (50 mM)	430
Li^+ (100 mM)	425
Rb^+ (50 mM)	450
Rb^+ (100 mM)	450

Table 2. Effect of various compounds on ATPase activity of *Mycoplasma mycoides* var. *Capri* membranes

The assay system contained 1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 50 mM-Tris, 5 mM- MgCl_2 , 50 mM of either choline chloride or NaCl and the inhibitor at the indicated concentration. Reaction was started by adding the membrane suspension previously incubated for 10 min with the same compounds.

Inhibitors	ATPase activity (nmol of P_i released/min per mg of protein)	
	Without Na^+	In the presence of Na^+
None	450	700
DCCD (50 μ M) (0.5 mM)	130	130
NBD (0.1 mM) (1 mM)	50	70
Ouabain (0.5 mM)	400	650
Vanadate (0.5 mM)	430	690

These findings concerning membrane ATPase activity do not provide a straightforward explanation of the Na^+ -dependent, ATP-consuming, K^+ -accumulation process described by Benyoucef *et al.* (1982). To gain insight into this mechanism, we studied the properties of Na^+ extrusion, with particular attention to the effects of K^+ .

Effects of K^+ on the energy-dependent Na^+ efflux

In the preceding paper, we reported that ' Na^+ -loaded cells', diluted in an Na^+ -free medium contain-

ing 1 mM-KCl, accumulated K^+ to a concentration of about 30 mM. In contrast, when these cells were resuspended in a medium containing 140 mM- Na^+ or more and 1 mM-KCl, i.e. in the presence of an Na^+ gradient, K^+ accumulated up to a concentration of 100 mM.

We therefore determine here the properties of the Na^+ extrusion process under these two different experimental conditions, and also examined the effect of K^+ addition.

Fig. 1 illustrates the time course of the changes in internal $^{22}Na^+$ when 'Na⁺-loaded cells' are diluted in a medium devoid of Na^+ . It appears that in starved cells, the cell $^{22}Na^+$ content seemed relatively stable as a function of time, since $^{22}Na^+$ only dropped by 20% in 10 min. In contrast, after addition of 10 mM-glucose to the cell suspension, the $^{22}Na^+$

Fig. 1. Na^+ extrusion by 'Na⁺-loaded cells' resuspended in an Na^+ -free medium

Fresh cells were previously depleted of their internal K^+ by 2 h incubation at 37°C in a buffer containing 50 mM-Mops, 17 mM-MgO, 120 mM-NaCl, 2 mM-MgCl₂ and 2 mM-H₃PO₄, pH 7.2. These 'Na⁺-loaded cells' (10–15 mg of cell protein) were resuspended in 0.5 ml of the same buffer supplemented with $^{22}Na^+$ as tracer (30 μ Ci/ml). After 20 min at 37°C, portions of this cell suspension were diluted 200–300-fold in the following Na^+ -free buffers, in the presence (●, ■ and ▲) or absence (O, □ and Δ) of 10 mM-glucose: in Mops/choline buffer in which NaCl was replaced by choline chloride, containing 1 mM-KCl (▲, Δ) or without KCl (●, O), or in Mops/Mg²⁺ buffer in which NaCl was replaced by MgCl₂ (■, □). At various intervals, samples of the cells were filtered and analysed for $^{22}Na^+$ content.

content decreases markedly from 100 mM down to 5 mM, with a half-time of 47 s. Surprisingly, Fig. 1 also shows that the presence of 1 mM-KCl in the medium did not accelerate the rate and extent of $^{22}Na^+$ loss, either in the presence or absence of glucose. Similar results were obtained when choline was replaced by Mg²⁺ as main cation (□, ■). The present results therefore indicate that under these experimental conditions, the efflux of Na^+ along an

Fig. 2. Na^+ extrusion by 'Na⁺-loaded cells' resuspended in an Na^+ -rich medium

Na^+ -loaded cells were prepared as described in the legend to Fig. 1 and resuspended (2 mg of cell protein/ml) in an Na^+ -rich buffer containing 100 mM-sodium phosphate, 40 mM-NaCl, 2 mM-MgCl₂ and $^{22}NaCl$ as tracer (15 μ Ci/ml) and supplemented with 1 mM-KCl (●) or without KCl (▲). After 20 min at 37°C, 10 mM-glucose was added and at intervals samples were filtered and analysed for $^{22}Na^+$ content. O represented the time course of $^{22}Na^+$ extrusion by cells pretreated with 5 μ M-FCCP before glucose addition. The inset shows results for $^{22}Na^+/Na^+$ exchange. 'Na⁺-loaded cells' (20 mg of cell protein/ml) were passively equilibrated with $^{22}Na^+$ in a buffer containing 100 mM-sodium phosphate, 40 mM-NaCl and 2 mM-MgCl₂, pH 7.2. After 20 min at 37°C, this cell suspension was diluted 400-fold in the same buffer but without tracer, and $^{22}Na^+$ cell content was analysed as a function of time in the presence (■) or absence (□) of 10 mM-glucose.

Na^+ concentration gradient is an energy-dependent process that does not require the presence of KCl in the medium.

In contrast, if the Na^+ efflux occurs against an Na^+ concentration gradient (Fig. 2), the Na^+ extrusion process requires the presence of KCl in the suspension medium. To measure cell Na^+ extrusion against an Na^+ concentration gradient, the following experimental procedure was used. ' Na^+ -loaded cells' were incubated in 100 mM-sodium phosphate buffer, containing $^{22}\text{Na}^+$ as a tracer, until transmembrane equilibrium was reached.

The changes in internal radioactivity were measured at various times after addition of glucose and/or K^+ . Since the specific radioactivity does not change during the course of the experiment, any change in internal $^{22}\text{Na}^+$ radioactivity corresponded to a change in the internal Na^+ concentration.

Fig. 2 shows a typical experiment illustrating the effects of glucose and/or K^+ on the intracellular Na^+ concentration: on addition of 10 mM-glucose, Na^+ is extruded if KCl is present in the external medium, but not in its absence.

This effect was highly specific for K^+ , since when RbCl replaced KCl, no Na^+ extrusion was observed. It should be recalled that under similar experimental conditions, whether Rb^+ equilibrated with $\Delta\psi_m$, K^+ accumulated well above the level predicted by $\Delta\psi_m$ (Benyoucef *et al.*, 1982). It is also noteworthy that the rate of Na^+ extrusion observed above is much lower than when Na^+ is extruded from cells resuspended in Na^+ -free medium ($t_{1/2}$ approx. 5 min instead of 47 s).

In addition, incubation of the cells with 5 μM -FCCP before glucose addition did not modify the capacity of the cells to extrude Na^+ , an observation that is taken into account when the effect of the transmembrane pH gradient on Na^+ and K^+ regulation is considered. An important finding was that in the absence of an energy source, no Na^+ extrusion was observed, whether KCl was present in, or absent from, the medium (results not shown).

The lack of changes in the internal Na^+ concentration observed in glycolysing cells resuspended in KCl-free medium does not mean that the membrane is completely impermeable to Na^+ . Indeed it can be observed that if cells loaded with $^{22}\text{Na}^+$ were diluted in a buffer containing unlabelled 200 mM- Na^+ , cell radioactivity falls rapidly (inset, Fig. 2). Taking account of this result and the result in the absence of KCl (Fig. 2), it can be concluded that, in a medium devoid of KCl, the Na^+ extrusion process only compensates for a rapid Na^+ entry.

The fact that the intracellular Na^+ concentration only decreases markedly when both glucose and K^+ were present clearly indicates that K^+ is essential for Na^+ extrusion against an Na^+ concentration gradient. This result is the complement of the

stimulatory effect exerted by Na^+ on K^+ accumulation described in the previous paper (Benyoucef *et al.*, 1982). Further, the observation that, in the absence of an Na^+ concentration gradient, Na^+ efflux occurs irrespective of the nature of the univalent cation species present in the external medium (K^+ , choline or Mg^{2+} ; Fig. 1) makes it likely that this Na^+ efflux takes place in exchange for protons, via an Na^+/H^+ antiport similar to that reported in other micro-organisms (for review, see Lanyi, 1979). This is why we attempted to estimate the contribution of proton movement to the coupling mechanisms between Na^+ and K^+ movements.

Fig. 3. Energy-dependent changes in the internal pH of ' Na^+ -loaded cells' resuspended in an Na^+ -free medium. General experimental conditions were as described in the legend to Fig. 1. (a) Shows changes in 9-aminoacridine fluorescence intensity. ' Na^+ -loaded cells' were resuspended at 200 $\mu\text{g}/\text{ml}$ in a Mops/choline buffer devoid of Na^+ and K^+ and containing 1.5 μM -9-aminoacridine. After a stable level of fluorescence was attained, 10 mM-glucose was added and the change in fluorescence intensity monitored at 455 nm ($\lambda_{\text{excitation}} = 400 \text{ nm}$). $\Delta F/F$ is percentage change in fluorescence intensity where F is the intensity before glucose addition. (b) Shows changes in [^{14}C]methylamine distribution. ' Na^+ -loaded cells' were resuspended at 1 mg/ml in a Mops/choline buffer devoid of Na^+ and K^+ and containing [^{14}C]methylamine (26 μM ; 38.4 Ci/mol). The [^{14}C]methylamine distribution was determined by filtration assay. The following additions were successively made, as indicated by the arrows: 10 mM-glucose, 5 μM -FCCP.

H⁺ movements during Na⁺ extrusion

The functioning of an Na⁺/H⁺-exchange mechanism implies that if an Na⁺ concentration gradient is set up across the membrane (e.g. Na_{in}⁺ > Na_{out}⁺), protons will move in the appropriate direction, thus generating a pH gradient (acidic inside). We monitored such a Δ pH change in *Mycoplasma* cells by using either fluorescence of 9-aminoacridine or [¹⁴C]methylamine distribution.

Fig. 3(a) illustrates the time course of the change in 9-aminoacridine fluorescence intensity when 'Na⁺-loaded cells' are diluted in a medium devoid of Na⁺ and K⁺. No fluorescence changes were detected in the absence of glucose. After addition of glucose a transient change in fluorescence intensity was observed. The initial quenching in fluorescence intensity ($\Delta F/F$ approx. -15%) indicated generation of Δ pH (inside acidic) across the membrane. After 5–6 min, the fluorescence reversed and levelled off ($\Delta F/F$ approx. +5%), indicating that the internal pH returned towards alkaline values.

Generation of a transient acid internal pH during Na⁺ efflux was confirmed by measuring the distribution of the weak base, [¹⁴C]methylamine, under the same experimental conditions. Fig. 3(b) shows the evolution of the internal pH after glucose addition to a suspension of 'Na⁺-loaded cells' in an Na⁺-free medium. In the absence of energy, the internal pH was around 7.0; after glucose addition, the pH fell to 6.6 in 4–5 min and then rose to 7.45. Addition of the protonophore FCCP at this stage abolished the preformed pH gradient. These data can be simply explained assuming that, after glucose addition, an inward proton movement took place together with the Na⁺ efflux observed under the same experimental conditions (Fig. 1). Since this H⁺ inward movement was only partly compensated by H⁺ extrusion by the Mg²⁺-dependent ATPase, the cytoplasm became acidic. After dissipation of the Na⁺ gradient due to the energy-dependent Na⁺ efflux, the only efficient system was the ATPase, which pumped protons outwards and led to final alkalization of the intracellular medium.

If the transient internal acidification is attributed to the functioning of such an Na⁺/H⁺ antiport, it may be inferred that such a change in pH does not occur in the absence of Na⁺ in the cell as well as in the external Na⁺. Indeed, when 'choline-cells' were diluted in a choline-buffered saline solution (Fig. 4a), i.e. in absence of any Na⁺ in the cuvette, the fluorescence rose immediately after glucose addition and reached a plateau value of $\Delta F/F = +5$ –6% in 1 min. Measurements of [¹⁴C]methylamine distribution confirmed this change (Fig. 4b). Thus, the observations reported on Figs. 3 and 4 clearly indicate the existence of an Na⁺/H⁺ antiport in *Mycoplasma* cells. Since it is known that in bacterial systems (Lanyi, 1979) such a mechanism is able to

Fig. 4. Energy-dependent changes in the internal pH of 'choline cells' resuspended in an Na⁺-free medium. General experimental conditions were as described in the legend to Fig. 3, but 'Na⁺-loaded cells' were replaced by 'choline cells', i.e. cells were devoid of internal Na⁺. (a) Shows changes in 9-aminoacridine fluorescence intensity. (b) Shows changes in [¹⁴C]-methylamine distribution.

extrude efficiently Na⁺ against an Na⁺ gradient, we attempted to evaluate the contribution of the Na⁺/H⁺ antiport to the mechanism of Na⁺ extrusion against a concentration gradient in *Mycoplasma mycoides* var. Capri.

Data in Figs. 5(a) and 5(b) respectively illustrate the changes in 9-aminoacridine fluorescence and [¹⁴C]methylamine distribution when 'Na⁺-loaded cells' are resuspended in a buffered saline solution containing 140 mM-Na⁺. In the absence of KCl from the cuvette, glucose induced stable quenching of fluorescence intensity (trace A in Fig. 5a, $\Delta F/F = -16\%$). This corresponded to acidification of the internal pH, which fell by 0.4 pH unit, as shown in Fig. 5(b). Thus, although no change in the internal Na⁺ concentration was observed under these experimental conditions (Fig. 2) these results indicate that the Na⁺/H⁺ antiport was functioning.

The H⁺ uptake by this antiport was constantly activated by the rapid Na⁺ reaccumulation and was just balanced by H⁺ extrusion by the Mg²⁺-dependent ATPase, leading to the stable acid internal pH.

Importantly, once the stable acid pH is set up, addition of 1 mM-KCl caused a rapid increase in

Fig. 5. Energy-dependent changes in internal pH of 'Na⁺-loaded cells' resuspended in an Na⁺-rich medium. General experimental conditions were as described in the legend to Fig. 2. (a) Shows changes in 9-aminoacridine fluorescence intensity. 'Na⁺-loaded cells' were resuspended at 200 μ g of cell protein/ml in a Mops/Na⁺ buffer (50 mM-Mops/17 mM-MgO/140 mM-NaCl/2 mM-MgCl₂/2 mM-H₃PO₄, pH 7.2) containing 1.5 μ M-9-aminoacridine. After a stable level of fluorescence intensity was attained, 10 mM-glucose was added (trace A). When a new stable level was attained, a pulse of 1 mM-KCl (trace B) or 1 mM-RbCl (trace C) was administered. Trace D represented the change in fluorescence intensity after glucose addition to cells previously incubated in the presence of 1 mM-KCl. (b) Shows changes in [¹⁴C]methylamine distribution. 'Na⁺-loaded cells' were resuspended at 1 mg/ml in a Mops/Na⁺ buffer containing [¹⁴C]methylamine (27 μ M; 38.4 Ci/mol). Then 10 mM glucose and 1 mM-KCl were added successively (●). ○, Cells were pretreated for 10 min with 5 μ M-FCCP before the experiment was started.

9-aminoacridine fluorescence intensity towards a positive level ($\Delta F/F$ approx. +9%), as shown in Fig. 5(a) (trace B). Accordingly, the release of previously accumulated [¹⁴C]methylamine indicated that the internal pH rose from 6.6 to 7.4 on KCl addition.

The same effect was not observed after addition of 1 mM-RbCl (Fig. 5a, trace C). This specificity of K⁺ correlates with two other observations: (a) K⁺, unlike Rb⁺, promotes Na⁺ extrusion against an Na⁺ gradient, and (b) K⁺, unlike Rb⁺, accumulates well above $\Delta\psi_m$ (Benyoucef *et al.*, 1981b, 1982).

It is also noteworthy that when these 'Na⁺-loaded cells' were preincubated in a buffer containing 140 mM-Na⁺ and 1 mM- or 10 mM-KCl, only a transient change in internal pH was observed on glucose addition (trace D). Finally, control experiments performed on FCCP-treated cells showed no changes in the internal pH after glucose addition (Fig. 5b). The significance of this transient pH change and of the K⁺-dependent alkalinization observed in Fig. 5 are considered in detail in the Discussion section.

Discussion

The results reported in the present paper further document the nature of the link between Na⁺ and K⁺ movements in *Mycoplasma* cells. Thus the measurements of the Na⁺ efflux against Na⁺ concentration gradient clearly indicate that under these experimental conditions, Na⁺ extrusion is a K⁺-dependent process. The data also provide evidence favouring the existence of an Na⁺/H⁺ exchange system capable of mediating part of the Na⁺ efflux across *Mycoplasma* membranes. The contribution of the two systems is taken into account below, when analysing the mechanism responsible for cation regulation in *Mycoplasma* cells.

Examination of Na⁺ and H⁺ movements led to the conclusion that, depending on the experimental conditions, part or all the Na⁺ efflux can be mediated by an Na⁺/H⁺ exchange system. Evidence supporting the presence of such an antiport has been reported for several micro-organisms (for review, see Lanyi, 1979). Two lines of experimental evidence lead to the conclusion that this also applied to *Mycoplasma* cells.

First, Figs. 1 and 3 indicate that in the absence of Na⁺ in the external medium, concomitantly with a rapid Na⁺ efflux, a transient acidification of the cell interior is observed and is followed by an alkalinization. This can be explained if it is assumed that: (a) initial H⁺ inward movement catalysed by an Na⁺/H⁺ antiport exceeds H⁺ extrusion by the Mg²⁺-dependent ATPase; (b) when the Na⁺ gradient is dissipated, the only system catalysing H⁺ movements is the Mg²⁺-dependent ATPase, which in turn leads to an alkalinization of the cell interior.

Secondly, in the absence of Na⁺ in the cells and the medium, there is no transient acidification (Fig. 4) but a direct alkalinization of the cell interior, which reflects the proton extrusion by the ATPase.

Lastly, turning to the conditions where 'Na⁺-loaded cells' are incubated in a medium with a high Na⁺ concentration but devoid of K⁺, the steady energy-dependent acidification observed (Fig. 5) is consistent with the functioning of an Na⁺/H⁺ antiport. In this case, since the internal Na⁺ content was kept constant by a rapid Na⁺ recirculation (Fig.

2, inset), the Na^+/H^+ antiport is continuously in activity and counterbalanced by H^+ extrusion by the ATPase and H^+ leaks.

From a functional point of view, it appears that the activity of the Na^+/H^+ antiport is not sufficient to promote net Na^+ movement against an Na^+ concentration gradient. As shown in Fig. 5, the net Na^+ efflux against an Na^+ concentration gradient requires the presence of K^+ .

This observation can be directly related to that reported in the preceding paper showing that K^+ accumulation was specifically stimulated by Na^+ under the same experimental conditions. A close link was evident between the K^+ and Na^+ movements against their respective gradients.

The experiments described in the present and preceding papers define a number of features that any model for K^+ cation regulation in *Mycoplasma mycoides* var *Capri* must accommodate. (1) In the absence of Na^+ , K^+ accumulates to the level expected from the transmembrane electrical potential amplitude, as predicted by the existence of a K^+ uniport system. (2) In the presence of Na^+ , K^+ accumulates above the level expected from the $\Delta\psi_m$ amplitude. (3) This Na^+ -stimulated K^+ active accumulation is $\Delta\psi_m$ -dependent. (4) The active Na^+ -dependent K^+ transport system is ATP-consuming. (5) Na^+ efflux against Na^+ concentration gradient specifically requires K^+ in the medium. (6) An Na^+/H^+ antiport is present that can mediate all or part of the Na^+ efflux.

We therefore consider K^+ transport and its link with Na^+ transport in terms of two types of mechanisms: those in which the energy necessary to accumulate one species is derived from the electrochemical gradient of other coupled species (particularly H^+) and those implying contribution of pumps (ATP-consuming systems). As regards the first type of mechanism, two models can be examined. The first was described by Sorensen & Rosen (1980), who observed that Na^+ -stimulated K^+ transport via the TrKA system in *Escherichia coli*, and suggested the possibility of an Na^+/K^+ co-transport. The energy for such transport could then be derived from the Na^+ electrochemical gradient established by the Na^+/H^+ antiport. Such a model, however, is unlikely in *Mycoplasma* cells, since we observed that Na^+/H^+ activity was unable to create the favourable $\Delta\mu_{\text{Na}^+}$ that serves as the immediate driving force for an Na^+/K^+ co-transport system.

Secondly, it seems unlikely that in *Mycoplasma* cells the coupling of Na^+ and K^+ fluxes resulted from coupling via an Na^+/H^+ antiport and a K^+/H^+ antiport of the type described by Bakker & Harold (1980), Brey *et al.* (1978) and Brey & Rosen (1979). Indeed it appears that, to explain the stimulation of the Na^+ efflux by K^+ or conversely the stimulation

of K^+ uptake by Na^+ , we would have to assume that the two antiports are linked via the proton chemical gradient. This gradient would in that case be consecutive to the H^+ entry via the Na^+/H^+ antiport, and would be used by the K^+/H^+ antiport. For K^+ accumulation, an exchange with protons is required. The effects of FCCP make the existence of a link via the proton gradient very unlikely. This protonophore in our strain selectively abolishes the transmembrane pH gradient across the membrane without changing the K^+ -dependent Na^+ efflux as well as the Na^+ -dependent K^+ uptake (Figs. 2, 3 and 5) (Benyoucef *et al.*, 1981a).

In addition to the specific considerations involved when considering the first type of mechanism, none of them predict the ATP consumption observed during Na^+ stimulation of K^+ uptake (Benyoucef *et al.*, 1982). Rather it seems that only ATP-driven cationic pumps can account for such a property. In this connection, Heefner *et al.* (1980) suggested the existence of a primary ATP-driven pump that exchanges Na^+ for H^+ or K^+ and requires alkaline pH. However, a prediction of this model is that in the presence of high K^+ concentration, an inward H^+ movement might be replaced by K^+ influx and therefore no cytoplasmic acidification would be observed. This is clearly not the case in our strain (Fig. 5a, trace D), since some degree of internal acidification occurred at a K^+ concentration as high as 10 mM, which corresponds to a value of about 50 times the K_m for K^+ accumulation (Leblanc & Le Grimellec, 1979a). Finally, when such an ATP-driven Na^+ pump functioned by way of an Na^+/H^+ exchange (i.e. in a K^+ -free medium) it failed to lower the intracellular Na^+ concentration against an Na^+ concentration gradient.

We are therefore dealing with the possibility that in *Mycoplasma mycoides* var. *Capri* cells, Na^+ and K^+ movements may be catalysed by a $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase of the type found in higher organisms. On the basis of our flux data, the most attractive model is the one schematically shown in Fig. 6: it includes an Mg^{2+} -dependent ATPase, a K^+ uniport system, an Na^+/H^+ antiport and an $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase, thus fulfilling all the requirements listed above and accounting for observations unexplained by other models. However, our studies of membrane ATPase activity might affect the validity of this model. In the first place, neither ouabain nor vanadate inhibited the ATPase activity in our membrane preparation. However, the lack of effect of such inhibitors, which did not alter either Na^+ efflux or K^+ influx (Leblanc & Le Grimellec, 1979b; results not shown) does not necessarily mean that an ATPase system similar to that found in animal cells is not operating. Indeed, it is not uncommon to find that established cation pumps display similar insensitivity to these inhibitors

Fig. 6. Na^+ , K^+ and H^+ movements in *Mycoplasma mycoides* var. *Capri*: an interpretation

Elements shown diagrammatically are the proton-translocating ATPase, an Na^+/H^+ antiport, a uniport system for K^+ , an Na^+ /phosphate exchange system and an ATP-consuming Na^+/K^+ exchange system [($\text{Na}^+ + \text{K}^+$)-dependent ATPase]. The broken line indicates the possibility of a coupling between an active pump for Na^+ and a uniport system for K^+ .

(Gutknecht, 1967; Barber, 1968). In contrast, the fact that K^+ did not affect enzyme activity, as well as a need for a high Na^+ concentration to stimulate the ATPase activity do not argue in favour of our model. One possible explanation is that during the membrane preparation procedure, either the protein responsible for Na^+/K^+ exchange was lost, or the affinities for univalent cations are markedly altered. Such an explanation has been proposed by Jinks *et al.* (1978) when describing the membrane ATPase in *A. laidlawii*, which is stimulated by low Na^+ concentrations. A different, although speculative, explanation might be that, although our results are consistent with functioning similar to that of an ($\text{Na}^+ + \text{K}^+$)-dependent ATPase, the coupling of Na^+ and K^+ fluxes may result from an interaction of two entities or subunits, for example an Na^+ -dependent ATP-driven pump and a K^+ uniport system. It is

conceivable that the interaction between these two entities is controlled by the electrochemical proton gradient $\Delta\mu_{\text{H}^+}$, or by one of its components (e.g. $\Delta\psi_m$). In that case K^+ stimulation would not be expected to take place in unsealed-membrane preparations. In this context, it would be interesting to test if resealing of our membrane preparation could lead to a resaturation of the activation of both ATPase activity and Na^+ efflux by K^+ .

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