Examination of the role of Na⁺ in the physiology of the Na⁺-dependent soil bacterium *Azotobacter salinestris*

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The growth of the Na⁺-dependent soil bacterium *Azotobacter salinestris* strain 184 was inhibited only 36% by 50 µM-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at alkaline pH, whereas other species of this genus were inhibited 80–90% under the same conditions. Growth of strain 184 at alkaline pH was inhibited 66% by 50 µM-monensin and 100% by monensin plus CCCP. The majority of the ATPase activity on everted membrane vesicles prepared from strain 184 grown at alkaline pH was sensitive to azide and *N,N'*-dicyclohexylcarbodiimide (DCCD), but ATPase was less sensitive to these inhibitors when Na⁺ was present. The respiratory activity of strain 184 was neither dependent on nor activated by Na⁺ and was unaffected by the antagonistic Na⁺-analogues K⁺ and Rb⁺. A Na⁺-dependent, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) supersensitive NADH oxidase was not present in strain 184. Na⁺ was required in the growth medium to promote optimal cell yields. Limiting the amount of Na⁺ available caused a lag phase in which cell viability was lost. Viability was maintained by the addition of Li⁺ or Mg²⁺ to Na⁺-limited medium, but only Li⁺ appeared to promote growth. K⁺ appeared to be a competitive inhibitor of a Na⁺/Li⁺ site required for cell growth. Rb⁺ was a more complex competitor and affected the final yield and the growth rate of strain 184. Rb⁺-tolerant mutants of strain 184 were selected and the majority of these were found to be defective in the Na⁺-dependent acid excretion normally observed with *A. salinestris*. Analysis of an acid over-producing strain showed that Rb⁺ appeared to be an uncompetitive inhibitor of Na⁺-dependent growth and in competition with Na⁺ as a promoter of acidification.

Introduction

Azotobacter spp. are well-known nitrogen-fixing bacteria found in soil and water and distributed world-wide (Thompson & Skerman, 1979). By far the most common species isolated from the soil is *A. chroococcum*. Strains of *Azotobacter* that were tentatively identified as *A. chroococcum*, but were dependent on Na⁺ for growth, were isolated by Page (1986). These bacteria account for ~5% of the aerobic nitrogen-fixing bacteria found in slightly saline soils of Western Canada (Page & Shivprasad, 1991).

The Na⁺-dependency of the *A. chroococcum*-like isolates is similar to that observed with marine bacteria (Reichelt & Baumann, 1974), whereby Mg²⁺ or Li⁺ can substitute for some of the required Na⁺ and promote optimal growth. This is not a trait shared by other azotobacters. Also, unlike other azotobacters, these

Na⁺-dependent strains are dependent on soluble iron for optimal growth (Page, 1987). Further studies revealed that iron was required for enzymic protection against toxic oxygen radicals (Page *et al.*, 1988). The strains were shown to be very sensitive to H₂O₂, catalase-negative and microaerophilic. However, they can grow and fix nitrogen aerobically (Shivprasad & Page, 1989). Based on these and other taxonomic tests Page & Shivprasad (1991) proposed that these Na⁺-dependent *A. chroococcum*-like bacteria be named *Azotobacter salinestris* sp. nov.

Although Na⁺ is required for growth of *A. salinestris*, little is known about its role in the physiology of these cells. This present study examines this question, using the roles of Na⁺ in marine and alkalophilic bacteria as instructive models. Gram-negative marine bacteria differ from their terrestrial counterparts in that they have a requirement for Na⁺ for cell viability and growth (Reichelt & Baumann, 1974), specifically in: (a) the accumulation of solutes (Niven & MacLeod, 1980), (b) osmotic stabilization of the cells and retention of solutes (MacLeod *et al.*, 1978); and (c) energy generation

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PCP, pentachlorophenol; RTC strain, clear, Rb⁺-tolerant strain; RTD strain, dense, Rb⁺-tolerant strain.

via Na^+ -motive force (Tokuda & Unemoto, 1985). In the latter activity, *Vibrio alginolyticus*, *V. costicola*, *V. parahaemolyticus* and the halotolerant bacterium *Ba₁* pump Na^+ out of the cells via a HQNO-sensitive, Na^+ -dependent NADH:quinone oxidoreductase (Ken-Dror *et al.*, 1986; Tokuda & Unemoto, 1984; Tsuchiya & Shinoda, 1985; Unemoto *et al.*, 1977). Na^+ influx is used to drive transport, motility and ATP synthesis (Chernyak *et al.*, 1983; Dibrov *et al.*, 1986; Skulachev, 1984). Cell growth and these activities are resistant to protonophorous conductors, particularly at alkaline pH (Chernyak *et al.*, 1983; Tokuda & Unemoto, 1983). Thus Na^+ performs a number of essential basic functions in the metabolism of marine bacteria.

The majority of terrestrial bacteria, on the other hand, may demonstrate Na^+ -stimulated growth or may use Na^+ /solute symport for the accumulation of specific solutes, but they do not demonstrate dependence on Na^+ for growth on all substrates (Lanyi, 1979; Reichelt & Baumann, 1974). Alkalophilic and alkali-tolerant bacteria demonstrate Na^+ -dependent growth at high pH where they must maintain an intracellular pH that is more acidic than the external pH (Booth, 1985). The exchange of internal Na^+ for external H^+ , via Na^+/H^+ antiport, is essential to maintain ΔpH for growth and Na^+ /solute symport activity (Booth, 1985; Grant, 1987). However, ATP generation in these cells is by H^+ -motive force and is sensitive to protonophorous conductors (Hicks & Krulwich, 1990).

Methods

Strains and culture conditions. This study used the type-strain of *Azotobacter salinestris* sp. nov., strain 184 (ATCC 49674; Page & Shivprasad, 1991). Other strains of *A. salinestris* used for comparative purposes were isolated and described previously (Page, 1986). The type strain of *A. chroococcum* 9043 and representative strains of *Azotobacter vinelandii* were obtained from the American Type Culture Collection. *A. vinelandii* strain UW is a University of Wisconsin subculture of strain ATCC 13705.

The cultures were routinely grown in Burk's minimal salts medium, containing 0.81 mM- MgSO_4 , 0.58 mM- CaSO_4 , 18 μM - FeSO_4 , 1.0 μM - Na_2MoO_4 , 1% (w/v) glucose and 0.11% (w/v) ammonium acetate in 5.0 mM-potassium phosphate buffer, pH 7.2. Media for *A. salinestris* also contained 0.8 mM- NaCl . Liquid cultures (40% of the flask volume occupied by the culture) were inoculated with approximately 5×10^6 cells ml^{-1} and were incubated aerobically at 28–30 °C with rotary shaking at 225 r.p.m. for 24 h.

Growth estimations. Culture growth was estimated by plate count assays, using plates of freshly prepared Burk's medium solidified with 1.8% (w/v) agar and protected from light to decrease the generation of toxic oxygen radicals in the medium (Page *et al.*, 1988). Viable count determinations varied by $\pm 20\%$, due to the microaerophilic nature of *A. salinestris* (Page *et al.*, 1988). Viable counts were made on exponentially growing cultures; older cultures gave very unreliable and low viable count estimates. Culture growth was also estimated by

protein determination as described previously (Page, 1986). All growth experiments were repeated at least twice and mean values are reported in the results. Protein determinations were very reproducible ($\pm 5\%$).

pH control and poisons. The pH of the Burk's medium containing 0.8 mM- NaCl was adjusted by altering the ratio of the phosphate salts present, with added NaOH to attain the more-alkaline pH values, and by the addition of 'Good's buffers' (Good *et al.*, 1966). The buffers used were PIPES (pH 6.0–7.0), HEPES (pH 7.0–8.0), TAPS (pH 8.0–9.0) and CAPS (pH 9.0–10.0). The pH of buffers was adjusted with NaOH. Changes in culture pH during incubation were measured with a Radiometer pH meter.

The following poisons were added to the growth medium or enzyme reaction mixtures from stocks made up in 95% ethanol: monensin, nigericin, *N,N'*-dicyclohexylcarbodiimide (DCCD), pentachlorophenol (PCP) and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was made up in dimethylsulphoxide and azide was from an aqueous stock. In all cases, the control culture or enzyme activity reaction (used as the 100% value) contained a concentration of solvent equal to that being added with the poison.

ATPase activity determination. *A. salinestris* was incubated in Burk's medium containing 50 mM-TAPS, pH 8.5, for 18 h. The cells from 1 litre of culture were harvested by centrifugation and resuspended to 1 g wet wt per 80 ml of 30 mM-Tris/acetate buffer, pH 7.8, containing 20% sucrose. Spheroplasts were formed as described by Kaback (1971) with the use of Tris/acetate buffer rather than Tris/HCl, as recommended by Bhattacharyya & Barnes (1976). The spheroplasts were lysed and vesicles formed as described by Kaback (1971) with Tris/acetate buffer used instead of phosphate buffer, to obtain vesicles with an everted topology (Bhattacharyya & Barnes, 1976). The final vesicle preparation (4–7 mg protein ml^{-1}) was suspended in 0.1 M-Tris/acetate buffer, pH 7.8, and stored in liquid nitrogen.

The ATPase in the *A. salinestris* membranes was activated by trypsin treatment as described by Bhattacharyya & Barnes (1976) with the modification that soya bean, rather than lima bean, trypsin inhibitor was used. ATPase activity was measured in a 1 ml assay containing (final concentrations): 3 mM-ATP; 50 mM-Tris/acetate buffer, pH 7.8; 4 mM- MgSO_4 ; and 60–125 μg membrane protein. The assay was incubated at 30 °C and the reaction rate was linear for 10 min. After 5 min incubation the reaction was stopped by the addition of 0.2 ml ice-cold 20% (w/v) trichloroacetic acid. The reaction mixture, a 1.5 ml microcentrifuge tube, was centrifuged for 10 min and the concentration of inorganic phosphate in the assay supernatant was determined (Chen *et al.*, 1956).

Respiratory activity. Membrane fragments for respiratory activity determinations were prepared from *A. salinestris* strain 184 or *A. vinelandii* strain UW that had been grown for 17–18 h in Burk's medium. The cells were harvested and resuspended in 50 mM-potassium phosphate buffer, pH 7.0, then DNAase and RNAase (0.05 mg ml^{-1} , final concentration) were added and the cells were broken in a French press [2000 lbf in^2 (about 13.8 MPa)]. Lysozyme (0.1 mg ml^{-1} , final concentration) was added and the mixture was incubated for 30 min at 20 °C and 60 min on ice. Cell debris was removed by centrifugation for 10 min at 2000 *g* in a Sorvall RC5-B centrifuge. Membranes were collected from the cell extract by centrifugation for 60 min at 40000 *g*. The membrane pellet was washed once and resuspended in 20 mM-Tris/HCl, pH 7.5.

Rates of oxygen uptake using isolated membranes and various electron donors were measured in a Clark oxygen electrode as described by Barnes (1973), but using Tris/HCl buffers rather than potassium phosphate buffers. Alternatively, NADH oxidase activity was determined spectrophotometrically in Tris/HCl buffer (Osborne *et al.*, 1972).

Results

Growth of *A. salinestris* strain 184 at alkaline pH

Strain 184 grew in Burk's medium over the range pH 6.2–10.0. However, these weakly buffered media had been adjusted by the cells to a final pH of about 7.5 in all cases where the initial pH had been greater than 7.5, and growth was delayed until this new pH was attained (data not shown). Addition of 20 mM Good's buffers to the Burk's medium failed to hold the initial pH to values >7.5. Addition of 50–100 mM Good's buffers held the initial pH within 0.5 pH unit from pH 8.0–10. The optimum pH for growth was 7.5–8.0 and there was no growth at pH 6.0 and pH values ≥9.5. HEPES buffer (pH 7.0–8.0) appeared to promote the best growth of strain 184, while PIPES (pH 7.0) and TAPS (pH 8.0) showed a slight inhibitory effect.

The production of ≥6 mM-succinic and 10 μM-citric and -glutamic acids by *A. salinestris* has been previously noted (Page, 1987) and acid production is one characteristic that distinguishes this species of *Azotobacter* from the others (Page & Shivprasad, 1991). The production of acid was promoted by increased concentrations of Na⁺ in the medium (Fig. 1). Acidification at NaCl concentrations >380 mM inhibited growth of *A. salinestris*, but acid continued to be formed by these non-growing cells until the NaCl concentration of the medium also inhibited metabolic activity. Therefore, the use of higher buffer strengths to hold the pH of the medium was thwarted by the secretion of acid by *A. salinestris* in response to the higher Na⁺ concentrations in the medium.

CCCP-resistant growth at alkaline pH

The growth of *A. salinestris* at pH 8.5 was inhibited only 36% by 50 μM-CCCP and was relatively insensitive to 100 μM-CCCP (Fig. 2). In comparison, growth of the type-strain of *A. chroococcum* was inhibited 91% by 50 μM-CCCP under the same conditions (Table 1) and demonstrated a response more typical of *Escherichia coli* (Tokuda & Unemoto, 1983). The tolerance of *A. salinestris* to CCCP was only observed at alkaline pH. Similarly, strain 184 was relatively insensitive at pH 8.5 to other proton gradient uncouplers such as PCP and nigericin, but it was fully inhibited by these agents at pH 6.5 (Table 1). This pattern of inhibition was very different from that observed with *A. chroococcum* (Table 1). The growth of strain 184 at pH 8.5 and pH 6.5 was inhibited 66% and 22%, respectively, by 50 μM-monensin, a Na⁺-ionophore. Growth of strain 184 was completely inhibited by 50 μM-monensin plus 50 μM-CCCP at pH 8.5. These data imply that *A. salinestris* has

Fig. 1. Acid production by *A. salinestris* in response to NaCl. The growth of strain 184 (□) and the final pH of the medium (■) were measured after 48 h incubation in Burk's medium containing NaCl.

Fig. 2. CCCP-insensitive growth of *A. salinestris*. The growth of strain 184 in the presence of CCCP was measured after 24 h incubation in Burk's medium buffered to pH 8.5 with 100 mM-TAPS (●) or buffered to pH 6.5 with 100 mM-PIPES (○).

Table 1. Sensitivity of *A. salinestris* 184 and *A. chroococcum* ATCC 9043 to membrane-activity poisons

Cultures were incubated for 24 h in Burk's medium containing 100 mM-PIPES, pH 6.5, or 100 mM-TAPS, pH 8.5, and the presence of the inhibitor concentration was noted. Cell growth was measured as the amount of protein ml⁻¹ and the means of replicate experiments (reproducibility ± 5%) are reported. 100% inhibition was not obtained because the residual cells were pigmented (melanized) which interfered slightly with the protein assay.

Addition	Concn (μM)	Growth inhibition (%)			
		<i>A. salinestris</i>		<i>A. chroococcum</i>	
		pH 6.5	pH 8.5	pH 6.5	pH 8.5
CCCP	50	97	9	96	91
	100	96	48	97	98
PCP	100	96	0	96	48
	300	96	0	96	91
Nigericin	10	96	22	96	93
	50	97	16	96	98
DCCD	10	29	5	94	91
	50	83	78	97	92
Azide	1000	97	18	95	31
	5000	96	56	96	85

Table 2. Effect of cations on the respiratory activity of *A. salinestris*

Substrate*	Addition (100 mM concn)	Specific respiratory activity† [nmol O ₂ (mg protein) ⁻¹ min ⁻¹]	
		<i>A. salinestris</i>	<i>A. vinelandii</i>
NADH	None	153	863
	NaCl	131	903
	RbCl	127	872
	KCl	123	
	LiCl	147	
NADPH	None	62	1259
	NaCl	64	1125
	RbCl	29	1225
Malate	None	41	1038
	NaCl	37	944
	RbCl	24	1109
Succinate	None	51	281
	NaCl	23	91
	RbCl	35	159
Lactate	None	0	
	NaCl	0	
	RbCl	0	

* 20 mM-malate, -succinate or -lactate and 2 mM-NADH or -NADPH were used.

† Means of duplicate tests ($\pm 8\%$ error) are shown.

a Na⁺-motive pump, other than Na⁺/H⁺ antiport, that operates at acidic and alkaline pH and that a Na⁺-cycle may allow the growth of these cells at alkaline pH in the presence of proton gradient uncouplers.

Respiratory activity in *A. salinestris*

The respiratory activity of isolated membranes of *A. salinestris* was examined (Table 2). The Na⁺-independent *A. vinelandii* strain UW was used for comparison because this strain does not produce a capsule, as does *A. chroococcum* ATCC 9043, which interferes with membrane isolation. *A. salinestris* had greatly reduced respiratory activity on all substrates when compared to the obligate aerobe *A. vinelandii*, a characteristic that is not unexpected of a microaerophilic bacterium (Krieg & Hoffman, 1986). The oxidation by *A. salinestris* of all substrates tested was neither dependent on nor enhanced by the presence of Na⁺.

Similar results were obtained when NADH oxidation by isolated membranes was assayed spectrophotometrically (data not shown). The NADH oxidase of *A. salinestris* was neither dependent on nor stimulated by NaCl. There also was no effect of KCl, LiCl or RbCl on the NADH oxidase activity of *A. salinestris* or *A. vinelandii*. NADH oxidase activity of *A. salinestris* and

Table 3. ATPase activity in membrane vesicles of *A. salinestris*

Experiments (a)–(c) were done with separate preparations of membrane vesicles.

Cation	Inhibitor	Specific ATPase activity* [nmol P _i (mg protein) ⁻¹ min ⁻¹]	Inhibition (%)
<i>Experiment (a)</i>			
None†	—	58.5	0
NaCl	—	50.6	14
KCl	—	48.9	16
LiCl	—	47.6	19
RbCl	—	59.9	0
<i>Experiment (b)</i>			
NaCl (10 mM)†	—	77.8	0
	DCCD (10 μM)	18.8	76
No NaCl	DCCD (10 μM)	2.5	97
<i>Experiment (c)</i>			
NaCl (10 mM)†	—	36.9	0
	Azide (250 μM)	25.0	32
	Azide (500 μM)	18.8	76
No NaCl	Azide (250 μM)	16.9	78
	Azide (500 μM)	4.7	87

* Means of duplicate tests ($\pm 10\%$ error) are shown.

† Control assays, with addition of solvent only, determine 100% activity. The ethanol solvent present in the DCCD series of assays routinely increased the ATPase specific activity.

A. vinelandii was inhibited 50% by 106 and 159 µM-HQNO, respectively. In comparison, the Na⁺-dependent NADH oxidase activity in membranes prepared from *V. alginolyticus* was stimulated ~5 to 10-fold by 100 mM-NaCl (Unemoto *et al.*, 1977) and was inhibited ~50% by 0.1 µM-HQNO (Tokuda & Unemoto, 1984).

ATPase activity in *A. salinestris*

Growth of strain 184 at pH 8.5 was relatively insensitive to azide, but was inhibited completely by azide at pH 6.5 (Table 1). Similarly, the ATPase inhibitor DCCD was less inhibitory at alkaline pH than at acid pH. The demonstration of ATPase activity using *A. salinestris* membrane vesicles was possible if the membrane vesicles were prepared in Tris buffer, pretreated with trypsin and assayed in the presence of MgCl₂ (4 mM final concentration). ATPase activity from *A. salinestris* grown at pH 8.5 was neither stimulated nor significantly inhibited by the addition of cations to the reaction mixture (Table 3). The ATPase activity was significantly sensitive to DCCD and azide, but was less sensitive to these poisons when NaCl was present in the assay mixture.

Table 4. Ion requirements and inhibitors of the growth of *A. salinestris*

Cells were incubated in Burk's medium for 16 h with NaCl and other cations added as noted. Results of a typical experiment are shown.

Na ⁺ concn (mM)	Other addition	Lag period (h)*	Mean generation time (h)†	Final yield (cells ml ⁻¹)
0.9	—	0	2.6	3.7 × 10 ⁸
0.7	—	1	2.7	2.3 × 10 ⁸
0.5	—	8	2.8	1.4 × 10 ⁷
0.3	—	12	3.0	3.0 × 10 ⁶
0.2	—	16	No growth	1.5 × 10 ⁶
0.3	Li ⁺ (7.5 mM)	3	2.3	3.2 × 10 ⁸
0.3	Mg ²⁺ (7.5 mM)	0	2.3	2.8 × 10 ⁸
0.9	K ⁺ (20 mM)	0	2.6	6.8 × 10 ⁷
0.9	Rb ⁺ (10 mM)	0	5.1	5.3 × 10 ⁶

* Estimated from turbidity measurements.

† Calculated from viable counts (± 20% error).

Growth promotion by Na⁺, Li⁺ and Mg²⁺

Limiting the amount of Na⁺ available for growth of strain 184 did not affect the growth rate, but caused an increased lag phase and a decreased final yield (Table 4). The addition of 7.5 mM-MgCl₂ or -LiCl to media containing 0.3 mM-NaCl decreased the lag phase time, promoted an optimal growth rate and near optimal cell yield (Table 4). Cells in the lag phase were not simply adjusting to the limited Na⁺ conditions, but also were losing viability. The initial viability of the culture containing 0.2 mM-Na⁺ decreased 7.4-fold after 16 h incubation. At the end of each lag phase in media containing 0.3, 0.5 and 0.7 mM-Na⁺ there was a 4-fold, 2-fold and 0-fold decline in viability, respectively. Addition of 5–20 mM-MgCl₂ to medium containing 0.2 mM-NaCl did not promote growth of the culture, but reduced the viability loss to 2-fold after 16 h incubation. Addition of 5, 7.5 and 10 mM-LiCl to medium containing 0.2 mM-NaCl promoted a 2-fold, 0.6-fold and 0-fold loss of viability, respectively. There was no growth in the media containing the 5–7.5 mM-Li⁺ additions, but a doubling of the viable count was observed with 10–15 mM-LiCl additions and the viable count increased 5-fold when 20 mM-LiCl was added. If 5–20 mM-LiCl was added to Na⁺-free medium, there was no growth and the viable count decreased 4-fold.

These results suggested similar but different roles for Mg²⁺ and Li⁺ when Na⁺ was limiting. Mg²⁺ appeared to be important in stabilizing the cells and decreasing cell viability loss. Li⁺ also stabilized the cells, but was more effective than Mg²⁺ and possibly was used with Na⁺ in promoting growth.

Fig. 3. (a) Inhibition of growth of *A. salinestris* by K⁺. The growth of strain 184 was measured after 24 h incubation in Burk's medium containing 0.9 mM-NaCl alone (Δ), with added 25 mM-LiCl (●) or with added 25 mM-MgCl₂ (○), or 0.3 mM-NaCl plus 25 mM-LiCl (□) or 0.3 mM-NaCl plus 25 mM-MgCl₂ (■). (b) Inhibition of growth of *A. salinestris* by Rb⁺. The growth of strain 184 was measured under the growth and ion-mixture conditions reported in Fig. (a).

Inhibition of growth by K⁺ and Rb⁺

It has been previously noted that K⁺ and Rb⁺ can substitute for Na⁺ (MacLeod *et al.*, 1978; MacLeod & Snell, 1950) and are antagonistic to growth of *A. salinestris* (Page, 1986). K⁺ affected only the final yield of cells, whereas inhibition by Rb⁺ was more complex, with the growth rate and the final cell yield affected (Table 4). It appeared that K⁺ was primarily competitive with a Na⁺/Li⁺ site (Fig. 3a). There was no competition provided by Mg²⁺ against K⁺ inhibition when Na⁺ was growth-limiting. However, Mg²⁺ provided protection against K⁺ inhibition when the Na⁺ concentration was higher (Fig. 3a). Rb⁺, on the other hand, was strongly competitive with Na⁺ (Fig. 3b). There was no protection provided by Li⁺ at high or low Na⁺ concentrations, although Mg²⁺ did provide protection when the Na⁺ concentration was higher.

If K⁺ was, in fact, affecting one Na⁺-dependent site that was essential for the growth of this strain, it seemed possible that one could calculate the nature of the K⁺-mediated inhibition of the growth of *A. salinestris* by treating a growth experiment like an enzyme reaction. This reaction was expected to demonstrate Michaelis-Menten-type kinetics whereby the culture protein yield

Table 5. Characterization of Rb^+ -tolerant strains

Representative strain	Percent-age of isolates	Acid prodn*	Growth in the presence of:†		
			$Na^+/Li^+‡$	$Na^+/Mg^{2+}§$	pH 8.5 ± CCCP
184	Wild-type	+	+	+	+
RTC-8	40	+++	+	+	+
RTD-15	18	+	—	+	+
RTD-19	4	+	+	—	+
RTD-94	17	+	+	++	+
RTD-30	20	—	+	+	—

* Overproduction (+++), Burk's medium acidified to pH < 5.5; underproduction (—), Burk's medium containing 100 mM-TAPS, pH 8.0–8.5, not acidified; normal (+), the latter medium acidified to pH 7.8–8.0, after 24 h incubation.

† Measured by protein assay.

‡ Burk's medium containing 0.3 mM-NaCl and 25 mM-LiCl. +, 100% of growth in medium containing 0.8 mM-NaCl; —, 15–30% of the (+) value.

§ Burk's medium containing 0.3 mM-NaCl and 25 mM-MgCl₂. Normal response (+) 50–70% of strain growth in 0.8 mM-NaCl (Page, 1986); stimulated growth (++) ≥ 100% of strain growth in 0.8 mM-NaCl; negative (—), 15–30% of the (+) value.

|| Burk's medium containing 100 mM-TAPS, pH 8.5, ± 25 μM-CCCP. +, 80–100% of strain growth in medium containing 0.8 mM-NaCl; —, 17–50% of the (+) value.

(Y) was dependent on the concentration of the Na^+ substrate (S) in the medium. A plot of Y vs S in the presence of increased K^+ concentrations resulted in a family of parallel curves where K^+ inhibition was easily overcome by increasing the concentration of Na^+ in the medium (a typical plot was shown previously by Page, 1986). A double reciprocal plot of these data resembled competitive inhibition of an enzyme.

Repeating the same experiments with Rb^+ as the inhibitor gave very different results. A plot of Y vs S showed that Rb^+ inhibition was overcome at higher Na^+ concentrations, but this occurred rapidly at first, then at a slower rate (a typical plot was shown previously by Page, 1986). A plot of $1/Y$ vs $1/S$ produced a series of lines where the inhibited reaction curves coincided below the $1/Y$ axis and resembled noncompetitive mixed inhibition of an enzyme. However, these lines did not have a common intercept with the uninhibited assay which indicated that more than one reaction was inhibited by Rb^+ .

Generation and analysis of Rb^+ -tolerant mutants

Strain 184 failed to grow after 3–4 d incubation on solid medium containing 25 mM- $RbCl$, but isolated colonies did appear after ≥ 5 d incubation. These colonies arose at a frequency of 4.0×10^{-8} per viable cell plated. One-hundred of these colonies were picked at random and

Fig. 4. Inhibition by Rb^+ of acidification of the medium by strain RTC-8. The final pH of Burk's medium containing $RbCl$ and 0.5 mM- (□), 0.9 mM- (■) and 1.2 mM- (○) $NaCl$ was measured after 24 h incubation.

maintained on medium containing $RbCl$. All of the strains were found to be Na^+ -dependent and they appeared to give the same growth response to limiting amounts of Na^+ as strain 184. The strains were not Rb^+ -dependent and were not truly resistant to Rb^+ because the growth of all the strains was inhibited by plating on media containing higher concentrations of $RbCl$. However, these strains were more tolerant of Rb^+ than strain 184, such that ~35% of the optimal protein yield was produced in the presence of 0.8 mM- Na^+ and 15 mM- Rb^+ after 24 h incubation.

The majority (60%) of these colonies were densely white in colour, as compared to the normal pinkish-tan colour of strain 184. These dense Rb^+ -tolerant (RTD) cells had a normal cell morphology, were actively motile and were filled with poly- β -hydroxybutyrate granules. The remainder of the Rb^+ -tolerant colonies were clear, without colour, contained cells with normal morphology that were actively motile, but contained not a trace of poly- β -hydroxybutyrate. All of the Rb^+ -tolerant colonies proved to be very difficult to maintain and lost viability quickly. This was particularly true of the clear Rb^+ -tolerant (RTC) strains which died after several transfers.

The RTC strains were found to excrete excess acid into the medium, lowering the pH to 5.5 after 24 h incubation (Table 5). This pH was lethal to the cells in liquid and solid media. Acid production by the RTC strains diminished when Rb^+ was present in the medium (Fig. 4). It appeared that Rb^+ was in negative competition with Na^+ as a promoter of acidification. In an attempt to treat the growth of this strain like a single enzyme reaction that was promoted by Na^+ and inhibited by Rb^+ , a double reciprocal plot was done as described previously. This plot resembled non-competitive inhibition of an enzyme.

The RTD strains were categorized into four phenotypes (Table 5). Strains defective in Li^+ -promoted-

Table 6. Ion requirements of natural isolates of *A. salinestris*Results shown are means $\pm$ SD of duplicate experiments for strains in each category.

Category	No. of strains	Percentage of Na ⁺ -sufficient growth*			Percentage of growth at pH 8.5†
		-Na ⁺ ‡	Na ⁺ /Li ⁺ §	Na ⁺ /Mg ²⁺	+CCCP¶
<i>A. salinestris</i>					
184-like	7	13 $\pm$ 14	125 $\pm$ 66	71 $\pm$ 19	88 $\pm$ 8
Mg-	8	18 $\pm$ 14	110 $\pm$ 44	22 $\pm$ 14	109 $\pm$ 42
Mg-/CCCP-	4	30 $\pm$ 21	105 $\pm$ 21	31 $\pm$ 22	35 $\pm$ 7
<i>A. chroococcum</i>	6	92 $\pm$ 19	92 $\pm$ 19	106 $\pm$ 43	18 $\pm$ 11

* Estimated as a percentage of the protein produced after 24 h incubation in Burk's medium containing 0.8 mM-NaCl.

† Estimated as a percentage of the protein produced after 24 h incubation in Burk's medium containing 100 mM-TAPS, pH 8.5.

‡ Burk's medium containing 0.3 mM-NaCl.

§ Burk's medium containing 0.3 mM-NaCl + 15 mM-LiCl.

|| Burk's medium containing 0.3 mM-NaCl + 15 mM-MgCl₂.¶ Burk's medium containing 100 mM-TAPS, pH 8.5, + 25 μ M-CCCP.

growth in medium containing limiting Na⁺, strains with altered Mg²⁺-promoted-growth or strains with normal ion stimulation, but unable to grow at alkaline pH $\pm$ CCCP, arose with approximately equal frequency. Defective Mg²⁺-promoted-growth in medium containing limiting Na⁺ was a rare event. In most cases Mg²⁺ promoted twice as much growth of the RTD strains in medium containing limiting Na⁺ as was obtained with strain 184. Defects in Li⁺- or Mg²⁺-promoted-growth were not a complete loss of function, but a diminished effect. In all cases there was real growth stimulation by these ions, but the amount of growth was 15–30% of that obtained with strain 184.

Variations in ion requirements of natural *A. salinestris* isolates

Since spontaneous mutants of *A. salinestris* were obtained with altered requirements for Li⁺ and Mg²⁺, albeit under selective pressure, it seemed possible that natural isolates might also vary in their ion requirements. Twenty-five strains were selected at random from a collection of *A. salinestris* and *A. chroococcum* strains. The *A. salinestris* isolates could be divided into three categories (Table 6). One group was like the type-strain, a second group was unaffected by the addition of Mg²⁺ to Na⁺-limited medium, and a third group was unaffected by Mg²⁺ and unable to grow well in alkaline medium containing 25 μ M-CCCP.

Growth of the *A. chroococcum* isolates was Na⁺-independent and unaffected by cation additions (Table

6). These strains were all sensitive to 25 μ M-CCCP at alkaline pH. Similar results were obtained with *A. chroococcum* ATCC 9043 and the *A. vinelandii* strains UW (ATCC 13705), O (ATCC 12518) and ATCC 12837 (data not shown).

Discussion

Initial studies with *A. salinestris* indicated its cells, unlike those of marine bacteria, did not lyse or lose UV-absorbing cell constituents in Na⁺-limited medium (Page, 1986). This is not surprising, considering that the differences in salt concentrations encountered in media for *A. salinestris* are too low to exert significant osmotic effects (MacLeod *et al.*, 1978). However, this present study shows that the cells do lose viability in Na⁺-limited medium. Viability is lost during a lag phase, the duration of which is dependent on the Na⁺ concentration of the medium. Once initiated, growth proceeds at a fixed rate then terminates, presumably as the Na⁺ concentration per cell becomes limiting. Viability can be maintained by addition of ≥ 7.5 mM-Li⁺ or -Mg²⁺ to Na⁺-limited medium. In the presence of small amounts of Na⁺, Li⁺ but not Mg²⁺ can shorten the lag phase and promote growth. Thus Li⁺ appears to be a functional analogue of Na⁺, whereas Mg²⁺ acts in a different manner. Similar results have been observed with *Alteromonas haloplanktis* where suboptimal Na⁺ concentrations cause a lag in growth and loss of viability (Gow *et al.*, 1981). The lag was overcome by increasing the Na⁺ available to the cells whereupon growth proceeded at a constant rate. Further studies with *Deleya aesta* and *A. haloplanktis* have shown that the lag phase is due to limited Na⁺-dependent nutrient accumulation (Berthelet & MacLeod, 1989). Li⁺ and Mg²⁺ were effective substitutes for Na⁺ to prevent solute efflux from cells suspended in Na⁺-free medium (MacLeod *et al.*, 1978). However, these ions were not effective in this capacity when they were present at low osmolalities and there is no evidence that *A. salinestris* must accumulate an essential metabolite, by Na⁺/solute symport, before growth is initiated (unpublished work).

It is possible that it is Na⁺ accumulation alone that is important before growth of *A. salinestris* is initiated. This would be limited by Na⁺ availability and the permeance of Na⁺ through the cell membrane. Similarly, the facultative alkalophile *Exiguobacterium aurantiacum* requires 0.9 mM-Na⁺ in the medium for growth at > pH 8.5 (Booth, 1985). The entry of Na⁺ into its cells is very rapid, but there is no evidence of any Na⁺/solute symport (Booth, 1985; McLaggan *et al.*, 1984).

Assuming Na⁺ enters cells of *A. salinestris*, what is it used for? A most striking feature of the metabolism of

A. salinestrus is the excretion of succinic and other organic acids (Page, 1986; Page & Shivprasad, 1991). Acid excretion is promoted by increased Na^+ concentrations, even in the absence of cell growth. Acid excretion is sufficient to lower the pH of alkaline media to a more neutral pH that will permit cell growth. This study shows that Rb^+ , an antagonist of Na^+ , appears to interfere with acid excretion. This is not due simply to growth inhibition, because acidification was promoted by Na^+ in the absence of cell growth as noted above. Rb^+ -tolerant strains were produced and 60% of these were either acid over-producers or under-producers. The RTC strains over-produced acid and demonstrated a growth response to Na^+ and Rb^+ that looks as if a single activity was being affected, with Rb^+ acting as a non-competitive inhibitor.

These results lead to the proposal that the organic acids excreted by *A. salinestrus* are transported out of the cell by Na^+ /solute symport. Na^+ /succinic acid efflux has not been reported in prokaryotes. However, endproduct symport of H^+ /lactic acid has been described as a means of generating protonmotive force in fermentative bacteria (Konings, 1985; Otto *et al.*, 1980). Succinic acid uptake has been reported to be Na^+ -dependent in *Micrococcus lysodeikticus*, Na^+ -stimulated in *E. coli* and Na^+ /solute symporters are reversible under some conditions (Lanyi, 1979). Endproduct efflux coupled to Na^+ is a plausible Na^+ pump, considering that *A. salinestrus* does not demonstrate the characteristics of the Na^+ -motive NADH oxidase of *V. alginolyticus*.

Growth of *A. salinestrus* at pH 8.5 was notably insensitive to protonophorous conductors, inhibited partially by monensin alone and totally by monensin combined with CCCP. CCCP-insensitive growth was a characteristic of the majority of natural isolates of *A. salinestrus* and was not a trait shared by *A. chroococcum* or *A. vinelandii*. However, at pH 6.5, growth of *A. salinestrus* was very sensitive to CCCP and relatively insensitive to monensin. This pattern of inhibition was very similar to that observed in *V. alginolyticus* (Dibrov *et al.*, 1986; Skulachev, 1984; Tokuda & Unemoto, 1983). These results imply that both H^+ -motive and Na^+ -motive cycles operate in *A. salinestrus*. The majority of the ATPase activity detected was sensitive to DCCD and azide, dependent on Mg^{2+} and activated by trypsin, characteristic of a H^+ -motive F-type ATPase (Pedersen & Carafoli, 1987). Such an ATPase has already been demonstrated in *A. vinelandii* (Eilermann *et al.*, 1971). However, there was DCCD- and azide-resistant ATPase activity in the presence of Na^+ , suggesting that an alternative, perhaps Na^+ -motive ATPase exists in *A. salinestrus*.

This study provides a hypothesis for further investigation of the physiology of *A. salinestrus*. The operation of

the proposed Na^+ /succinate efflux pump needs to be demonstrated as well as the Na^+ -motive ATPase. Questions are raised about the nature of the Na^+ -dependent site that is competitively inhibited by K^+ , the site that will accept Li^+ as a substitute for Na^+ and the nature of the interaction of Mg^{2+} with cells of *A. salinestrus*. Is this latter ionic interaction dispensable, as suggested by the examination of natural isolates of *A. salinestrus*? These questions and others are currently under investigation.

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