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Proton Motive Force and Na^+/H^+ Antiport in a Moderate Halophile†

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The influence of pH on the proton motive force of *Vibrio costicola* was determined by measuring the distributions of triphenylmethylphosphonium cation (membrane potential, $\Delta\psi$) and either dimethyloxazolidinedione or methylamine (osmotic component, ΔpH). As the pH of the medium was adjusted from 5.7 to 9.0, the proton motive force steadily decreased from about 170 to 100 mV. This decline occurred, despite a large increase in the membrane potential to its maximum value at pH 9.0, because of the loss of the pH gradient (inside alkaline). The cytoplasm and medium were of equal pH at 7.5; membrane permeability properties were lost at the pH extremes of 5.0 and 9.5. Protonophores and monensin prevented the net efflux of protons normally found when an oxygen pulse was given to an anaerobic cell suspension. A Na^+/H^+ antiport activity was measured for both Na^+ influx and efflux and was shown to be dissipated by protonophores and monensin. These results strongly favor the concept that respiratory energy is used for proton efflux and that the resulting proton motive force may be converted to a sodium motive force through Na^+/H^+ antiport (driven by $\Delta\psi$). A role for antiport activity in pH regulation of the cytosol can also explain the broad pH range for optimal growth, extending to the alkaline extreme of pH 9.0.

Vibrio costicola is a moderately halophilic bacterium which requires 1 M NaCl for optimal growth (13) and lyses in media of low osmotic strength (9). Lysis can be prevented by salts other than NaCl, but there is a specific Na^+ requirement for carrier-mediated transport (24). Understanding the specific requirement for Na^+ in transport requires knowledge of the electrochemical ion gradients in *V. costicola*.

A transmembrane proton motive force ($\Delta\bar{\mu}_{H^+}$) may be established in various microorganisms by proton efflux by using respiratory energy, light energy in photosynthetic organisms or extreme halophiles, or the energy from ATP hydrolysis (15, 36), resulting in a membrane potential ($\Delta\psi$, interior negative) and an osmotic component (ΔpH, interior alkaline) such that in millivolts, $\Delta\bar{\mu}_{H^+} = \Delta\psi - 60 \cdot \Delta pH$, at 30°C (30). Energy may be released from the osmotic component by proton-symport mechanisms in medium more acidic than the internal pH (pH_i); at a higher pH of the medium (pH_0), the cytosol is more acidic than the medium, so $\Delta\psi$ must compensate for the inverse pH gradient.

In certain bacteria, a sodium motive force may be formed by several mechanisms, resulting from the outward-directed movement of Na^+. First, the energy of the proton motive force may be converted to a sodium motive force through Na^+/H^+ antiport activity as found in *Alteromonas haloplanktis* (33), *Halobacterium halobium* (26), alkalophilic bacilli (29), *Mycoplasma mycoides* (6), and others, including *Escherichia coli* (4, 44) (for a review, see reference 25). In addition to a role in energy coupling, antiport activity may be involved in regulation of cytosolic pH (34). Second, in *Klebsiella aerogenes*, efflux of Na^+ through the membrane is driven by the Na^+-requiring membrane-bound enzyme oxaloacetate decarboxylase (12). The methylmalonyl-coenzyme A decarboxylase of *Micrococcus lactilyticus* (14, 18) and the glutaconyl-coenzyme A decarboxylase from the anaerobe *Acidaminococcus fermentans* (8) appear to have similar properties. Third, a Na^+-stimulated ATPase activity may be linked to Na^+ efflux, as in *M. mycoides* (5, 6), *Acholeplasma laidlawii* (20), and *Streptococcus faecalis* (17). Fourth, in *Vibrio alginolyticus* in alkaline medium, a Na^+ efflux system driven directly by respiration (without intermediary H^+ efflux) and insensitive to protonophores can establish a sodium motive force and $\Delta\psi$ (43). As a final example, halorhodopsin was considered to be an outward-directed Na^+ pump in *H. halobium* but is now identified as a light-driven inward-directed chloride

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pump (38). The sodium gradient in *H. halobium* is thus established solely through Na^+/H^+ antiport activity (38).

As part of a larger study on the energetics of Na^+-dependent carrier-mediated transport and salt tolerance in *V. costicola* (24), we measured the effect of pH_0 on the proton motive force and identified a Na^+/H^+ antiport activity. We present evidence here that the antiporter is involved in regulation of the cytosolic pH.

MATERIALS AND METHODS

Bacterial cultures. *V. costicola* NRCC 37001 was grown in 500-ml Erlenmeyer flasks containing 100 ml of medium and was shaken on a rotary shaker at 30°C. The growth medium included 1% (wt/vol) Difco Proteose Peptone no. 3 (Difco Laboratories, Detroit Mich.), 1% (wt/vol) tryptone (Difco), and 1.0 M NaCl (final pH 7.6) (13).

At the end of the exponential phase of growth (16 to 18 h), bacteria at a cell density of 1 to 1.5 mg (dry weight) per ml were washed twice with a salt solution of 1.0 M NaCl, 8 mM KCl, 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, and 50 mM Tris-hydrochloride buffer (final pH 7.2) and were suspended in this solution to the densities indicated. For salt solutions of different pH values, MES [2-(*N*-morpholino)ethanesulfonic acid]–KOH (50 mM) was used for pH values lower than 7.6, and Tris-hydrochloride (50 mM) was used for pH values higher than 7.2.

For investigating Na^+/H^+ antiport, bacteria were washed and incubated in 1 mM Tris (pH 7.5) containing 1.0 M KCl, 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, and 50 mM KSCN. The bacterial suspension (4 mg [dry weight] per ml) was stored on ice before being used; it could be kept like this for up to 6 h without any measurable change in activity.

Measurement of intracellular volume. The space in a packed cell pellet penetrated by [^{14}C]inulin (0.2 mg/ml, 2.5 μCi/mg), [^{3}H]raffinose (0.1 mM, 0.54 μCi/μmol), or [^{14}C]urea (0.1 mM, 1.5 μCi/μmol) was compared with the total space measured gravimetrically (28). Radioactive markers were added to 5 ml of bacterial suspension (about 2 mg [dry weight] per ml) in the salt solution used to wash the bacteria. After 15 to 20 min of incubation at room temperature, tubes were centrifuged (10 min, 7,700 × *g*), and the radioactivity of the extracts and supernatants was determined as described (19).

The dry weight of bacteria was determined after drying to constant weight at 62°C, ashing for 6 h at 550°C, and correcting for the weight of salt in the ashed material. No difference in dry weight was found if cells were dried in a vacuum oven.

Measurement of ΔpH. Intracellular pH was measured by the distribution between the intracellular space of the bacteria and the medium of a weak acid, [^{14}C]DMO (5,5-dimethyl-2,4-oxazolidinedione), or of a weak base, [^{14}C]methylamine (37). Bacteria (1.1 mg [dry weight] per ml) were incubated with constant shaking at 30°C for 20 min in solutions of different pH values before adding either DMO (2.6 μM, 55.4 μCi/μmol) at $pH_0 \leq 7.6$ or methylamine (8.92 μM, 56 μCi/μmol) at $pH_0 \geq 7.6$. After 20 min of further incubation, 1.5-ml samples of the bacterial suspension were centrifuged in a Microfuge (2 min, 13,000 × *g*), and the pellets were extracted with 1 M $HClO_4$. The radioactivity of the extracts and that of the supernatants diluted 10 times with 1 M $HClO_4$ were determined in 5 ml of Aquasol. Cells at pH_0 7.2 were incubated with urea (0.1 mM, 1.5 μCi/μmol) to determine the intracellular plus extracellular space in the cell pellets. It was assumed that intracellular space would not change with pH_0. The pH of each supernatant was measured with an Orion digital pH meter. pH_i was calculated for DMO by the formula of Mitchell et al. (32) with 6.3 as the pK_a of DMO (1); for methylamine, pH_i was calculated by the method of Maloney et al. (28). The ΔpH represents the difference between pH_i and the external pH.

Measurement of Δψ. Δψ was determined at 30°C by measuring the accumulation of a lipophilic cation, triphenylmethylphosphonium ($TPMP^+$), in the presence of the anion tetraphenylboron (TPB^-). The bacteria (0.5 mg [dry weight] per ml) were incubated for 20 min in the salt solution before the addition of [^{3}H]$TPMP^+$ (10 μM, 10 μCi/μmol) and 2 μM TPB^-. Samples of 0.5 ml were then filtered on 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.) which had previously been soaked in 10 μM $TPMP^+$ (41) and immediately washed with 5 ml of a solution identical to the incubation solution, except that it contained no $TPMP^+$ or TPB^-. The results were erratic if the wash was omitted. Nonspecific accumulations of $TPMP^+$ by the cells and filter were corrected for by subtracting the counts associated with cells filtered immediately upon addition of [^{3}H]$TPMP^+$ in the absence of TPB^-. The filters were solubilized at least 12 h in 5 ml of Aquasol before radioactivity was measured. Δψ was calculated from the Nernst equation, where $\Delta\psi = 60 \log (TPMP^+_{in}/TPMP^+_{out})$ (36). The total proton motive force was calculated by replacing Δψ and ΔpH by their values in the equation $\Delta\bar{\mu}_{H^+} = \Delta\psi - Z\Delta pH$ where $Z = 60$ mV at 30°C (30).

Metabolic changes of different markers. Bacteria were incubated with labeled compounds for 20 min at the optimal pH for the accumulation of DMO (5.5) or methylamine (9.2), and with urea and raffinose as indicated above. The bacteria were then centrifuged, and the supernatant (S_1) was retained. The pellets were treated with 0.1 ml of *n*-butanol for 15 min at 80°C. Next, 0.3 ml of water was added, and the mixture was held at 80°C for an additional 15 min (19). The suspension was recentrifuged, and the second supernatant (S_2) was removed. The supernatants (S_1 and S_2) were examined by thin-layer chromatography as follows. (i) Urea: silica gel G, 250 μm thick (Mandel Scientific Co., Ltd., Rockwood, Ontario); solvent, chloroform-methanol-water (70:50:10, vol/vol). (ii) Raffinose: silica gel 60, 250 μm thick (BDH Chemicals Canada, Ltd., Toronto, Ontario), previously treated with 4 g of $NaHSO_3$ in 80 ml of water and 120 ml of ethanol, dried in air, and activated at 100°C for 30 min (11); solvent, ethyl acetate-methanol-acetic acid-water (60:15:15:10, vol/vol). (iii) DMO: silica gel G, 250 μm thick (Mandel); solvent, chloroform-methanol (90:10, vol/vol). (iv) Methylamine: cellulose, 100 μm thick (Brinkmann Instruments Canada, Ltd., Rexdale, Ontario); solvent, isopropanol-ethanol-water-hydrochloride (75:75:45:5, vol/vol). The R_f values of authentic labeled compounds urea, raffinose, DMO, and methylamine were 0.62, 0.07, 0.77, and 0.57, respectively, as shown by autoradiography.

Evidence for Na^+/H^+ antiport. The reaction vessel described by Patel and Agnew (35) was connected via a gas-tight seal to a H^+ electrode. Two hypodermic needles inserted through the fluted butyl-rubber serum stopper of the sampling port permitted a continuous flow of N_2 to circulate through the reaction vessel. Bacterial suspensions were injected through the serum stopper (5 ml, 0.4 mg [dry weight] per ml), and 100 μg of carbonic anhydrase was added (33). Samples were stirred magnetically at room temperature for 30 to 60 min, during which the pH was adjusted to 7.5 by injections of oxygen-free HCl or KOH. This adjustment was done to obtain bacterial cells with minimal or no transmembrane pH difference.

Solutions added (50 μl) contained the test compound (KCl, choline chloride, NaCl, or LiCl) at 2.5 M, each prepared with 0.4 mM $MgSO_4$ and 0.2 mM KH_2PO_4. The solutions were saturated with nitrogen or oxygen in closed 60-ml serum bottles, and the pH was adjusted with HCl or KOH to match the pH of the cell suspension.

Effects of the uncouplers CCCP (carbonylcyanide *m*-chlorophenylhydrazone) and TCS (3,3′,4′,5-tetrachlorosalicylanilide) and that of monensin were studied by incubating the bacteria in their presence for 20 min at 30°C at a final concentration of 20 μM before placing the suspensions in the reaction vessel. Because *V. costicola* can use ethanol as a source of electrons in respiration, the inhibitors were added as methanolic solutions.

For a control, 20 μl of *n*-butanol was added to a 5-ml suspension of cells, which was then heated at 85°C for 30 min. Traces of DNase and RNase were added to reduce the viscosity before placing the treated cells in the reaction vessel.

Optimum pH for growth. *V. costicola* was grown in the complex medium already described with a NaCl content of 1 M and with 0.06% (wt/vol) polypropylene glycol as antifoam. A 3-liter water-jacketed vessel (Pegasus Industrial Specialties, Ltd., Searborough, Ontario) containing 2 liters of medium was maintained at 30°C. Saturation of the medium with oxygen was ensured by supplying oxygen (100%) at 130 ml/min and dispersing with a Vibromixer (Pegasus). The pH was regulated automatically by injections of NaOH (2 N), with variations of less than 0.05 pH units. Inocula consisted of 20 ml of a late-logarithmic-growth-phase culture (24 mg, dry weight). Generation times were calculated from measurements of optical density at 660 nm measured hourly for 8 h. Separate growth curves were obtained for each pH tested.

Reagents. [2-^{14}C]DMO, [^{14}C]urea, [*methyl*-^{3}H]$TPMP^+$, [*carboxyl*-^{14}C]inulin, [^{3}H]raffinose, and Aquasol were purchased from New England Nuclear of Canada, Lachine, Quebec, Canada. [^{14}C]methylamine was supplied by Amersham, Oakville, Ontario, Canada. Carbonic anhydrase (2,100 U/mg), tetraphenylboron, DNase, RNase, and CCCP were from Sigma Chemical Co., St. Louis, Mo. TCS was from Fisher Scientific Co., Nepean, Ontario, Canada. Monensin was a gift from Eli Lilly Canada, Inc., Toronto, Ontario.

RESULTS

Intracellular volume. The effectiveness of different probes in estimating the intracellular volume of *V. costicola* was compared by measuring the space in a packed cell pellet to which a series of labeled compounds had access. As a reference, the total water space in the pellet was measured gravimetrically. Urea penetrated the cytoplasm completely, whereas both raffinose and inulin occupied about 59 ± 7% of the available space in the pellet. [^{14}C]urea penetrations gave less variable results than the gravimetric method and were preferred. Raffinose was chosen instead of inulin as a nonpermeant marker because it is a smaller molecule and may pass more freely through the outer membrane of *V. costicola*. Although raffinose and inulin gave comparable results after growth and suspension in solutions containing 1 M NaCl, raffinose may be preferred if the cytoplasmic membrane is shrunken from the outer wall, as happens upon suspension in 3 or 4 M NaCl solutions (24). Sucrose could not be used since *V. costicola* was able to grow with sucrose as the sole carbon and energy soûrce (data not shown). The measurement of intracellular volume calculated from the difference between the space available to urea and raffinose was 1.81 ± 0.22 μl/mg (dry weight; nine separate measurements).

Thin-layer chromatography of supernatants and cell extracts revealed that urea, raffinose, methylamine, and DMO (used in a later section) were not metabolized. We also found that *V. costicola* was incapable of utilizing inulin as a growth substrate.

Proton motive force. When cells were incubated in air, both components of $\Delta\bar{\mu}_{H^+}$ were affected by the pH_0 of the medium (Fig. 1). The chemical potential (60 · ΔpH) declined to 0 as pH_0 was increased to 7.5, whereas $\Delta\psi$ steadily increased to a maximum value of 160 to 170 mV at pH_0 9.0. Respiration rates were highest also at pH_0 9.0 (manuscript in preparation). However, this increase in $\Delta\psi$ was insufficient to compensate for the loss of the pH gradient (inside alkaline), and the total $\Delta\bar{\mu}_{H^+}$ declined from 168 to 95 mV as the pH_0 was adjusted from 5.5 to 9.0. *V. costicola* is capable of maintaining a fairly constant pH_i, which increased from 6.9 to 8.0 when the pH_0 was raised from 5.1 to 9.2.

Accumulation of the weak acid, DMO, or the weak base, methylamine, takes place over a wide range of pH_0. *V. costicola* lyses at pH_0 values below 5.0 and greater than 9.5; lysis is manifested by an immediate clearing of the turbid suspension. At pH_0 7.5, when the pH gradient is 0 according to methylamine distribution, a small amount of DMO uptake occurs (equivalent to 120% penetration). This low level of uptake is probably caused by nonspecific binding of the label.

Measurements of $\Delta\psi$ were conducted with $TPMP^+$ in the presence of a hydrophobic anion,

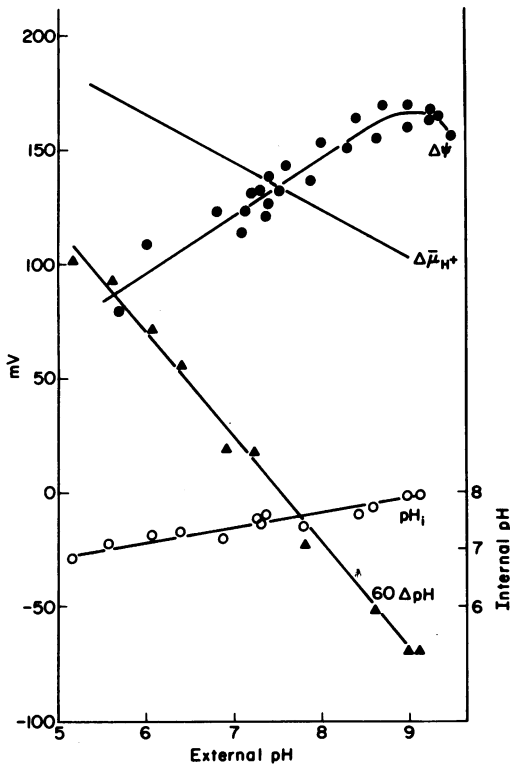

FIG. 1. Influence of pH_0 on the proton motive force of *V. costicola*. Δψ was measured with [^{3}H]TPMP$^+$ plus TPB$^-$, and ΔpH was measured with [^{14}C]DMO and [^{14}C]methylamine as described in the text. The composition of the buffer systems (which contain 1 M NaCl) is described in the text. The chemical potential (60 · ΔpH) and Δψ were summed to calculate $\Delta\bar{\mu}_{H^+}$ in mV.

TPB$^-$. The anion promoted an initial rapid uptake of TPMP$^+$, but in the steady state (10 to 20 min) almost no effect of tetraphenylboron was evident (data not shown). Certain bacteria require the counterion for the uptake of TPMP$^+$ (19, 21); others do not (16). Tetraphenylphosphonium was not used because it gave very high nonspecific binding as determined by rapid filtrations after mixing of cells and label. Separate experiments showed that uptake of ^{86}Rb in valinomycin-treated cells (3) could not be used to measure Δψ since valinomycin appears to be ineffective with *V. costicola* (results not shown).

Inhibitor effects on Δψ. Inhibition of respiration in this obligate aerobe by KCN (10 mM) or *N*-ethylmaleimide (10 mM) caused a dramatic decline in Δψ (80 to 92% inhibitions). The ionophores (20 μM) nigericin, monensin, CCCP, and TCS caused inhibitions in Δψ after 20-min treatments of 97, 60, 68, and 72%, respectively (manuscript in preparation). All of these effects were measured with a pH_0 of 8.5 to 8.8.

Na^+/H^+ antiport. *V. costicola* can use the energy of respiration for proton efflux at alkaline pH_0. When the bacteria were incubated anaerobically in a solution containing 1.0 M KCl to prevent lysis (9) and to maintain equal osmolarity to the growth medium, an oxygen pulse was followed by a rapid acidification of the medium (Fig. 2). The incubation medium also contained KSCN. The freely permeable SCN^- ions can rapidly compensate for the charge created by movement of ions across the membrane and thus prevent the development of an appreciable Δψ (31). As the oxygen was consumed, protons were reabsorbed by the bacteria, and the pH_0 returned to its original value. The acidification of the medium in response to an oxygen pulse was prevented by protonophores (CCCP, TCS), monensin, and butanol.

If, however, the bacteria were preincubated with NaCl before the oxygen pulse was given, a

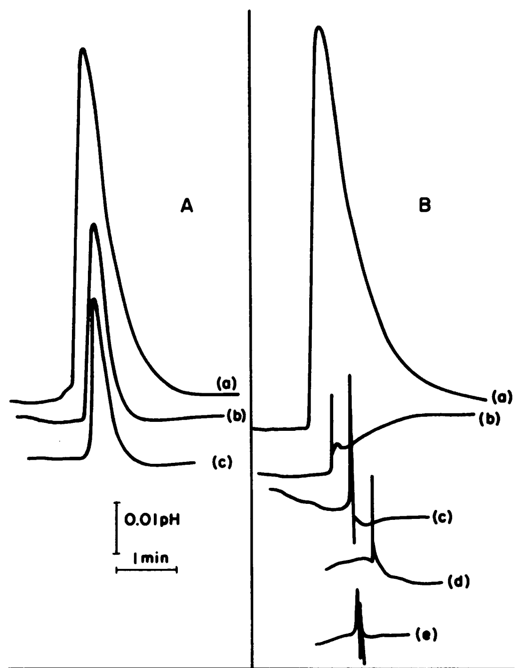

FIG. 2. Influence of NaCl and inhibitors on respiration-linked proton efflux in *V. costicola*. Proton efflux was measured with a H^+ electrode as the increase in acidity resulting from injections of oxygen-saturated 2.5 M KCl to anaerobic cell suspensions. (A) Anaerobic cell suspensions were incubated in salt solution (pH 7.5) containing 1 M KCl and either no NaCl (a), 25 mM NaCl (b), or 50 mM NaCl (c). (B) Anaerobic cell suspensions in the same salt solution (pH 7.5) containing 1 M KCl (but no NaCl) were preincubated 20 min at 30°C with inhibitors before the oxygen pulse: (a) methanol control, 47 mM; (b) CCCP, 20 μM; (c) TCS, 20 μM; (d) monensin, 20 μM; (e) butanol treatment as described in the text.

smaller response which decayed more rapidly was noted. The half-times for decay (Fig. 2A) were 3.2 s (curve a), 1.8 s (curve b), and 1.4 s (curve c) for NaCl concentrations of 0, 25, and 50 mM, respectively. Previously, it was shown that respiration rates were similar at external concentrations of NaCl of 0 and 1.0 M (24). These results suggest that a Na^+/H^+ antiporter is functioning, causing the reabsorption of protons coupled with an exit of Na^+. The rate of antiport activity increased as a function of the concentration of Na^+ present in the anaerobic preincubation medium (no transmembrane Na^+ gradient is expected in anaerobic cells [33, 45]) to a maximum rate at 40 mM NaCl.

Na^+/H^+ antiport can also be demonstrated by injecting NaCl anaerobically into cells suspended in oxygen-free 1.0 M KCl (Fig. 3A). In this case, the presumed influx of Na^+ was accompanied by a pronounced efflux of protons. The antiport seems specific for Na^+ ions since little or no acidification of the medium occurred upon injections of LiCl, KCl, or choline chloride. Further convincing evidence for Na^+/H^+ antiport was provided by inhibitor studies (Fig. 3C through E). After incubations of *V. costicola* with CCCP, TCS, or monensin, no appreciable NaCl-induced acidification of the medium occurred. Since the ionophores were injected as methanolic solutions, we showed as a control that the same quantitative results were obtained in cell suspensions to which an equivalent amount of methanol was added (Fig. 3B). Finally, the results with butanol-treated (killed) cells (Fig. 3F) show that none of the responses was nonspecific or caused by pH mismatch of solutions.

A role for Na^+/H^+ antiport in regulation of the cytosolic pH was tested by measuring the effect of NaCl on the accumulation of methylamine (Table 1). At a pH_0 near neutrality, methylamine did not accumulate, as predicted from results shown in Fig. 1. However, a large Na^+-dependent accumulation of methylamine occurred at pH 8.6, supporting a role for the antiporter in acidifying the cytoplasm at alkaline pH_0. Moreover, in aerobic cells similar data were obtained, although pH_i was somewhat more alkaline when respiration-driven H^+ efflux was occurring (Table 1).

Several of the observations made suggest that *V. costicola* should grow well in an alkaline medium. As predicted from measurements of respiration rates, $\Delta\bar{\mu}_{H^+}$, and the Na^+/H^+ antiporter, *V. costicola* grew at maximum rates over a wide range of pH_0 extending well into the alkaline range (Fig. 4).

DISCUSSION

The results indicate that $\Delta\psi$ is greatest at alkaline pH_0 values (pH 9.0), when the pH

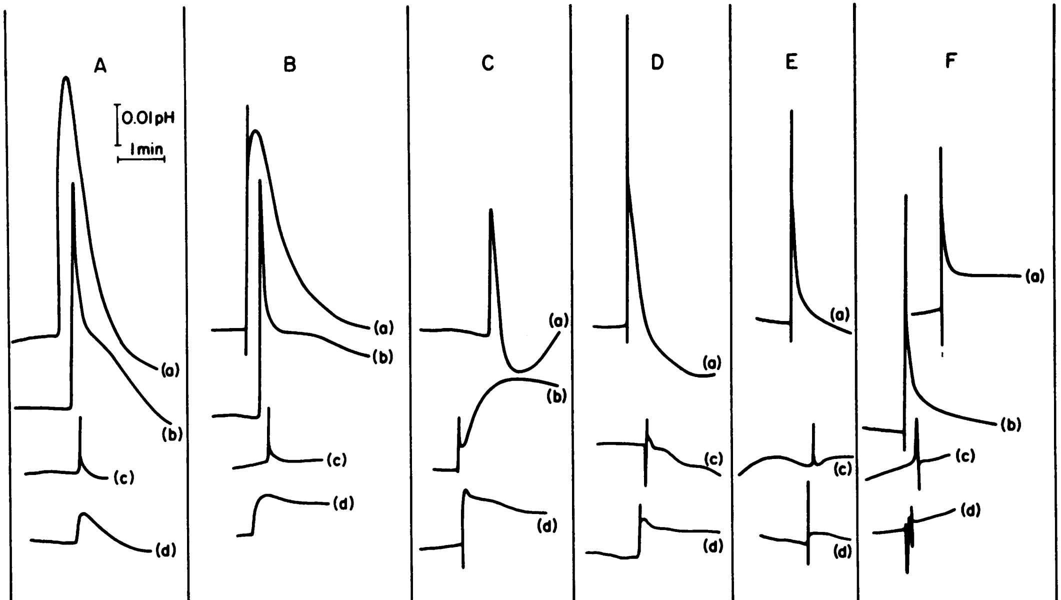

FIG. 3. Effect of ionophores and butanol on Na^+/H^+ antiport in *V. costicola*. Proton efflux was measured with a H^+ electrode as an increase in acidity resulting from injections of 25 mM concentrations of NaCl (a), LiCl (b), KCl (c), or choline chloride (d) to anaerobic cells suspended in a salt solution of pH 7.5 (1 M KCl, no NaCl). (A) Control cells; (B) methanol control, 47 mM; (C) CCCP, 20 μM; (D) TCS, 20 μM; (E) monensin, 20 μM; (F) butanol treatment (see legend to Fig. 2).

TABLE 1. Effect of NaCl on pH_i of *V. costicola*[a]

Gas phase	Salts medium	pH_0	pH_i	ΔpH
Nitrogen	KCl	7.11	7.11	0[b]
		8.58	7.95	0.63
	NaCl	7.00	7.00	0
		8.63	7.53	1.10
Air	KCl	6.98	6.98	0
		8.58	8.58	0
	NaCl	6.93	6.93	0
		8.64	7.77	0.87

[a] *V. costicola* (10 mg [dry weight] of cells) was suspended in 5 ml of the salt solution described in the text and containing either 1 M NaCl or 1 M KCl (with NaCl omitted). After storage of the cell suspensions for 30 min under N_2 or air, [^{14}C]methylamine uptake was allowed to occur for 20 min. Samples of 1.5 ml were centrifuged (13,000 × *g*, 2 min), and the distribution of radioactivity between the cells and medium was determined.

[b] 0, ΔpH (inside acidic) was not detected with the methylamine probe.

gradient is reversed (inside acidic). We have shown separately (manuscript in preparation) that respiration rates are also greatest at pH_0 9. Direct evidence for a respiration-linked efflux of protons was obtained by showing an increase in the acidity of the medium when anaerobic cells were given an oxygen pulse. This proton efflux in response to a respiratory pulse was sensitive to the protonophores CCCP and TCS, as well as monensin, as expected for reagents which abolish pH gradients. These results were obtained at such a pH_0 that no transmembrane pH gradient existed (before the respiratory pulse). In general, H^+ efflux coupled to cation/proton antiport can account for establishment of $\Delta\psi$ when pH_0 is more alkaline than the cytoplasmic pH. Such a mechanism has been demonstrated for *Bacillus alcalophilus* (29) and proposed for certain methanogenic bacteria (19), nitrifying bacteria (23), and others (34).

The proton motive force of *V. costicola* was measured as a function of pH_0. At a pH_0 of 7.5, the chemical potential (60 ΔpH) disappeared (inside alkaline), leaving $\Delta\psi$ as the sole component of $\Delta\bar{\mu}_{H^+}$. At pH_0 values above 7.5, the pH gradient reversed, becoming acidic inside. Under these conditions (pH_0 8.5 to 8.8), $\Delta\psi$ was dissipated by protonophores, by inhibitors of respiration, and by monensin, which normally serves to facilitate Na^+/H^+ exchange (2). The sensitivity of $\Delta\psi$ to these reagents illustrates the importance of respiration-driven proton efflux in establishing $\Delta\psi$.

A Na^+/H^+ antiporter was identified in *V. costicola*, which may function to convert the $\Delta\bar{\mu}_{H^+}$ established by respiration-linked H^+ efflux to a sodium motive force. Such an interconversion of electrochemical ion gradients through Na^+/H^+ antiporter activity has been shown in several microorganisms, including *H. halobium* (27) and *E. coli* (4). Previously, it was shown that *V. costicola* maintains a cytoplasmic concentration of K^+ above that in the medium and a cytoplasmic concentration of Na^+ below that in the medium (10, 40). In the absence of a ΔpH (inside alkaline), the driving force for the Na^+/H^+ antiporter is likely to be $\Delta\psi$, where the stoichiometry of H^+ influx to Na^+ efflux must be greater than 1. An electrogenic antiporter functioning at alkaline pH_0 has been detected in membrane vesicles of *E. coli* (39) and *H. halobium* (26), but direct evidence has not been obtained yet for *V. costicola*.

Energy coupling at alkaline pH_0 is different in *V. costicola* and *V. alginolyticus*. Both of these moderate halophiles have maximal $\Delta\psi$ at about pH_0 9 (42). However, evidence has been presented that at alkaline pH_0, *V. alginolyticus* has a respiration-dependent primary Na^+ extrusion system, which gives rise to $\Delta\psi$ and a Na^+ chemical gradient (43). In contrast to these results, in *V. costicola* $\Delta\psi$ is very sensitive to protonophores, and Na^+ does not stimulate rates of oxygen consumption greatly, not even at pH_0 8.5 (this study and unpublished results). Thus, a primary respiration-linked proton efflux to establish $\Delta\bar{\mu}_{H^+}$ coupled to Na^+/H^+ antiporter activity is required to explain the data for *V. costicola* adequately.

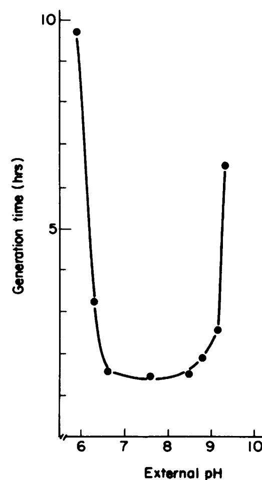

FIG. 4. Growth of *V. costicola* as a function of pH_0. Growth studies were conducted in a fermentor equipped with pH control, using the complex medium described in the text. Both pH and growth (optical density) were monitored hourly.

In addition to a role in energy coupling, a second function possible for antiporters is in the regulation of intracellular pH (34). Na^+/H^+ antiport has been most clearly implicated in studies of a non-alkalophilic mutant of *B. alcalophilus* (22) and in *E. coli* studies in which mutants defective in this activity are also unable to grow in alkaline medium (46, 47). Our results with *V. costicola* show that Na^+ is involved in pH regulation, which is correlated with an ability to grow optimally at pH_0 well into the alkaline range, i.e., with both aerobic and anaerobic cell suspensions maintained a more constant pH_i in the presence of Na^+ than in its absence. Since some Na^+ always contaminates the Na^+-depleted medium, these results are probably even less dramatic than what would be observed in the complete absence of Na^+. The possibility has yet to be tested that *V. costicola* contains a K^+/H^+ antiporter, which has been implicated in pH homeostasis in *E. coli* (7).

V. costicola is a neutrophile according to the classification of Padan et al. (34) since it grows within the pH range of 5.5 to 9.0. The optimum pH_0 for growth extends from pH_0 6.5 to 9.0, during which the pH_i increases from 7.25 to 7.9. The $\Delta\psi$ also increases dramatically. By coupling cation/proton antiport to $\Delta\bar{\mu}_{H^+}$, the organism is capable of optimal growth into the alkaline pH range when the pH gradient is reversed (inside acidic). This, in addition to the known ability of *V. costicola* to grow over a wide range of salt concentrations (13), should provide well for its survival during changing environmental conditions.

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