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Growth of a Marine *Vibrio alginolyticus* and Moderately Halophilic *V. costicola* Becomes Uncoupler Resistant When the Respiration-Dependent Na^+ Pump Functions

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The growth of *Vibrio alginolyticus* and *V. costicola*, which possess respiration-dependent Na^+ pumps, was highly resistant to the proton conductor carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), in alkaline growth media, even though the membrane was rendered permeable to H^+. The pH dependence of CCCP-resistant growth was similar to that of the Na^+ pump. In contrast, *Escherichia coli* ML308-225 showed neither Na^+ pump activity nor CCCP-resistant growth, even when grown in alkaline, Na^+-rich media. These results suggest that certain bacteria possess the Na^+ pump and are thus able to grow under the conditions where H^+ circulation across the membrane does not take place. Moreover, *V. alginolyticus* growing in the presence of CCCP maintains normal levels of internal K^+, Na^+, and H^+. The Na^+ pump, therefore, makes the growth of these organisms resistant to CCCP by maintaining the intracellular cation environments.

In accordance with the chemiosmotic theory of Mitchell (20, 21), bacteria generate an electrochemical potential of H^+ (a proton motive force) across the membrane by the active extrusion of H^+. It is well established that various energy-dependent reactions are coupled to the influx of H^+ moving down its electrochemical potential. This type of energetics, involving H^+ circulation across the membrane (energy-generating H^+ extrusion and energy-consuming H^+ influx), is common to all bacteria. Thus, once the H^+ circulation is disrupted, by proton conductors, for example, most bacteria are unable to perform various energy-dependent reactions and, finally, cannot survive.

On the other hand, we have discovered a primary Na^+ pump which is coupled to the electron transport of *Vibrio alginolyticus* (27, 28). Since the Na^+ pump has a pH optimum in the alkaline region and has little activity at pH 6.5 and below, *V. alginolyticus* demonstrates two different types of energetics depending on the external pH. At acidic pH, only the proton motive force is generated as the immediate result of respiration, and it plays a central role in energetics. The generation of the Na^+ electrochemical potential at pH 6.5 is performed by a Na^+/H^+ antiport system and, hence, is secondary to the generation of the proton motive force. At alkaline pH, however, the generation of the Na^+ electrochemical potential is a primary process, performed by the Na^+ pump. Therefore, significant differences are observed in the sensitivity to CCCP of certain energy-linked processes at acidic and alkaline pHs. For example, CCCP added to Na^+-loaded cells in NaCl solution at pH 8.5 does not collapse the membrane potential ($\Delta\psi$), but leads to the generation of a pH gradient (ΔpH, inside acid) of a magnitude similar to that of the $\Delta\psi$. On the other hand, neither a $\Delta\psi$ nor ΔpH is generated at pH 6.5 in the presence of CCCP (28). The active extrusion of Na^+ at pH 6.5 is performed only by the Na^+/H^+ antiport system and is completely inhibited by CCCP, whereas the Na^+ pump performs CCCP-resistant Na^+ extrusion at pH 8.5. Thus, the active transport of amino acids and sucrose, driven by the electrochemical potential of Na^+, is resistant to CCCP at pH 8.5, but not at pH 6.5. From these and other unpublished data, we hypothesized that H^+ circulation may not be required for the growth of *V. alginolyticus* at alkaline pH, since a sodium motive force takes its place for membrane-linked energy-requiring reactions. To our knowledge, the present paper contains the first report that a certain class of bacteria, possessing the Na^+ pump, are able to grow in the absence of H^+ circulation.

MATERIALS AND METHODS

Bacteria and media. The bacterial strains used were *V. alginolyticus* 138-2, *V. costicola* NCMB 701, and *E.*

coli ML308-225 *lacI lacZ*. The last strain was obtained from H. R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J. Each bacterium was grown aerobically at 37°C on a medium containing 0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4, 0.2% glucose, and 0.5 M NaCl (complex medium) for *V. alginolyticus*; 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 10 mM $MgSO_4$, 0.2% glucose, and 1.0 M NaCl for *V. costicola*; and 1% tryptone, 0.5% yeast extract, 0.2% glucose, and 0.3 M NaCl for *E. coli*. Where indicated, *V. alginolyticus* was also grown on a synthetic medium (25) containing 0.3 M NaCl and 1% glycerol. Besides those listed above, each medium contained the specified buffer, 2-(*N*-morpholino)ethanesulfonic acid (MES), HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], or *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), at a concentration of 50 mM, and the pH was adjusted with NaOH. The cells were harvested at the late-logarithmic phase of growth.

Determination of growth rate. The growth of cells was monitored by measuring the turbidity at 600 nm with a Perkin-Elmer spectrophotometer (model 35). A portion of preculture was inoculated into 5 ml of fresh medium in a 20-ml Monod test tube to give a turbidity of about 0.02, and aeration was attained by agitation. The addition of CCCP dissolved in dimethyl sulfoxide was made at the early logarithmic phase of growth (turbidity at 600 nm, about 0.05). The growth rates presented were determined before the turbidity reached about 0.3. The changes in the medium pH during this period were not significant, and the given value of medium pH represents the initial pH.

$^{22}Na^+$ extrusion by Na^+-loaded *E. coli*. The method used for *V. alginolyticus* (27, 28) was slightly modified. K^+ depletion and Na^+ loading of *E. coli* were performed at 25°C in 50 mM diethanolamine-hydrochloride (pH 9.3)–0.4 M NaCl containing 0.1 mg of chloramphenicol per ml. The Na^+-loaded cells suspended in 0.4 M NaCl containing 0.1 mg of chloramphenicol per ml and 50 mM of either Tricine-NaOH (pH 8.5) or MES-NaOH (pH 6.0) were equilibrated with $^{22}NaCl$ (carrier free) for 2 to 3 h on ice. The extrusion of $^{22}Na^+$ against the Na^+ concentration gradient was determined by the filtration method (28).

H^+ flow across membranes of *V. costicola* induced by oxygen pulse. An anaerobic suspension of *V. costicola* in a water-jacketed vessel (Radiometer, TTA 60 titration assembly) was pulsed with oxygen at 25°C by the addition of 0.1 ml of air-saturated 1.2 M salt solution, and the medium pH was monitored by a glass electrode (G2040C) attached to a pH meter (Radiometer, PHM84) with a reference calomel electrode (K701) as described elsewhere (28). The changes in the medium pH induced by the oxygen pulse were recorded by a Hitachi 056 recorder.

Determination of $\Delta\psi$ and ΔpH. Generations of $\Delta\psi$ (negative inside) and ΔpH (acidic inside) by *V. alginolyticus* were examined at 25°C from the equilibrium distribution of [^{3}H]tetraphenylphosphonium$^+$ (TPP^+; 19 μM, 157 μCi/μmol) and [^{14}C]methylamine (21 μM, 44 μCi/μmol), respectively. The accumulation of these probes was determined by filtration as mentioned previously (28). The cells were preincubated at 25°C for 5 min in 50 μl of specified assay medium. Since the assays were also performed in growth media, chloramphenicol (0.1 mg/ml) was included in all of the assay media to prevent growth during assays. The addition of chloramphenicol had no effect on $\Delta\psi$ and ΔpH generated by Na^+-loaded cells in NaCl solution. When the synthetic medium was used for assay, Mg^{2+} was omitted to increase the permeability of the outer membrane to TPP^+. On the other hand, TPP^+ was permeable to the cells in the complex medium, and the addition of EDTA had no effect on a magnitude of $\Delta\psi$. The addition of Mg^{2+} did not alter the magnitude of ΔpH generated by Na^+-loaded cells in CCCP-containing NaCl solution. The assay was started by the addition of a radioactive probe. Where indicated, CCCP was added before the start of the assay. At given time intervals, the uptake was terminated by the addition of 2 ml of the respective salt solution at room temperature and by filtration with cellulose acetate filters (Schleicher & Shuell; pore size, 0.45 μm). The filters were washed once, and the radioactivities were determined. The values of the internal water space of cells grown on the synthetic and complex media were 3.3 (25) and 4.8 (29) μl/mg of cell protein, respectively. These values were used to calculate the steady-state concentration gradients of probes across membranes. $\Delta\psi$ was determined from the Nernst equation, $\Delta\psi = 59\log(TPP^+)_{in}/(TPP^+)_{out}$. The internal pH was calculated from the distribution of methylamine (pK = 10.62) as described elsewhere (24). ΔpH ($pH_{in} - pH_{out}$, in units), the chemical potential of H^+ (59 ΔpH, in millivolts), and the total proton motive force ($\Delta p = \Delta\psi - 59\Delta pH$, in millivolts) were then calculated.

Determination of K^+ and Na^+. The intracellular concentrations of K^+ and Na^+ were determined in *V. alginolyticus* growing on the synthetic medium containing 50 mM MES-NaOH (pH 6.5) or Tricine-NaOH (pH 8.5) as a buffer. CCCP at a final concentration of 5 μM was added to a portion of culture when the turbidity at 600 nm reached about 0.2. From time to time, samples (0.5 to 5 ml) of culture containing 0.1 to 0.3 mg of protein were filtered through cellulose acetate filters. As a blank, filtration was also performed with fresh medium without cells. The filters were washed twice with 2 ml of 0.4 M choline chloride–10 mM Tris-hydrochloride (pH 7.0). The K^+ and Na^+ contents were analyzed after extraction with 5% trichloroacetic acid by flame photometry with a Perkin-Elmer 403 atomic absorption spectrophotometer as described elsewhere (22).

Determination of protein. Protein was determined as described by Lowry et al. (19), using bovine serum albumin as a standard.

Materials. [^{3}H]TPP^+ (bromide salt) was a generous gift from H. R. Kaback. [^{14}C]methylamine-hydrochloride and $^{22}NaCl$ were purchased from New England Nuclear Corp. (Boston, Mass.). CCCP was obtained from Sigma Chemical Co. (St. Louis, Mo.); MES, HEPES, and Tricine were from either Nakarai Chemicals Ltd. (Kyoto, Japan) or Wako Pure Chemical Industries Ltd. (Osaka, Japan). Polypeptone was a product of Daigo Eiyo (Osaka, Japan). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, Mich.).

RESULTS

Effect of CCCP on the growth of *V. alginolyticus*, *E. coli*, and *V. costicola*. The growth of *V. alginolyticus* on the complex medium at pH 6.5,

where the H^+ pump but not the Na^+ pump functions, was immediately and almost completely terminated by the addition of 5 μM CCCP (Fig. 1A). On the other hand, the growth at pH 8.5, where the Na^+ pump has a maximum activity, was only slightly inhibited by 30 μM CCCP, and a considerable growth was observed even in the presence of 50 μM CCCP (Fig. 1B). The minimum inhibitory concentrations of CCCP were 4 μM at pH 6.5 and higher than 80 μM at pH 8.5. The membranes of *V. alginolyticus* become completely permeable to H^+ at both pH values when 5 to 10 μM CCCP is added (25, 28). Furthermore, as discussed below, the proton motive force generated in the complex medium is collapsed by CCCP at both pH values. Therefore, *V. alginolyticus* is able to grow at pH 8.5 even if H^+ is permeant to its membrane.

The growth rates of *E. coli* on the medium containing 0.3 M NaCl were determined at pH 6.5 and 8.5 as a function of CCCP concentration (Fig. 2A). In contrast to the results obtained at pH 6.5 with *V. alginolyticus* (data not shown) or *V. costicola* (Fig. 2B), CCCP had little effect on the growth rate of *E. coli* at concentrations

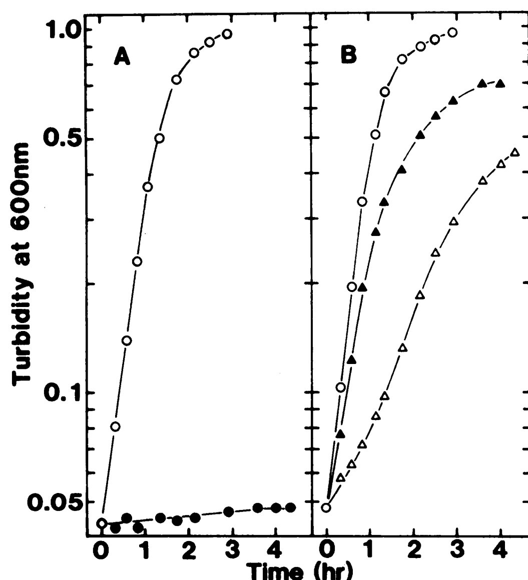

FIG. 1. Growth of *V. alginolyticus* on media containing CCCP. The growth of *V. alginolyticus* at 37°C on the complex medium containing 50 mM of either (A) MES-NaOH (pH 6.5) or (B) Tricine-NaOH (pH 8.5) was followed by measuring the turbidity at 600 nm as described in the text. When the turbidity reached about 0.045 (0 time), CCCP was added to give final concentrations of 0 (○), 5 (●), 30 (▲), and 50 (△) μM. The growth rates at pH 6.5 were 2.93 and 0 generations per h in the presence of 0 and 5 μM CCCP, respectively. The growth rates at pH 8.5 were 3.33, 2.55, and 1.12 generations per h in the presence of 0, 30, and 50 μM CCCP, respectively.

lower than critical values, which were higher at pH 8.5 than at pH 6.5 (Fig. 2A). The MICs of CCCP were about 20 and 35 μM at pH 6.5 and 8.5, respectively; these values were similar to the concentration of CCCP necessary to make membranes permeable to H^+ at the respective pHs (data not shown). We ascribe the difference in the MICs at pH 6.5 and 8.5 almost entirely to the difference in the critical concentrations seen at the two pHs. We conclude that *E. coli* is unable to grow when the membrane becomes permeable to H^+.

The growth of *V. costicola* at pH 6.5 was sensitive to CCCP (Fig. 2B). The MIC for CCCP was about 2 μM. However, when the pH of the medium was raised to 8.5, *V. costicola* was able to grow even in the presence of about 20 μM CCCP. The membrane of *V. costicola* at pH 8.5 became completely permeable to H^+ in the presence of 2 μM CCCP (data not shown). We conclude that *V. costicola* is able to grow at pH 8.5 in the absence of H^+ circulation.

Effect of pH on CCCP-resistant growth of *V. alginolyticus*. The growth of *V. alginolyticus* on the complex medium with or without 5 μM CCCP was examined in more detail over the pH range of 6.0 to 9.0. The growth rates determined did not change substantially over the pH range tested in the absence of CCCP, but in the presence of CCCP growth was strongly dependent on the medium pH (Fig. 3). The fastest growth in the presence of 5 μM CCCP was obtained at pH 8.5. When the medium pH was lower than 7.0, the cells were unable to grow in the presence of 5 μM CCCP. Such a pH dependence of the CCCP-resistant growth showed a good agreement with the pH dependence of the Na^+ pump (28). When the synthetic medium was employed instead of the complex medium, the growth in the presence of 5 μM CCCP also occurred at pH 8 to 9.

Na^+ pump activity in *E. coli* and *V. costicola*. Although no primary Na^+ extrusion system is known in *E. coli*, it is possible that such a system is induced in this nonhalophilic bacterium. Since the Na^+ pump of *V. alginolyticus* functions at alkaline pH, we thought it worthwhile to examine *E. coli* grown on an alkaline, Na^+-rich medium. The cells obtained after growth at pH 8.5 on the medium containing 0.3 M NaCl were depleted of internal K^+ and loaded with Na^+ to observe the bulk extrusion of Na^+ against its concentration gradient. As in *V. alginolyticus* (26, 27), the bulk extrusion of Na^+ in *E. coli* was initiated immediately by the addition of K^+ as a counterion at both pH 6.0 and 8.5 (Fig. 4). In the absence of K^+, only about 15% of loaded Na^+ was extruded during a 10-min incubation. CCCP (50 μM) completely inhibited the active extrusion of Na^+, not only at pH 6.0, but also at pH

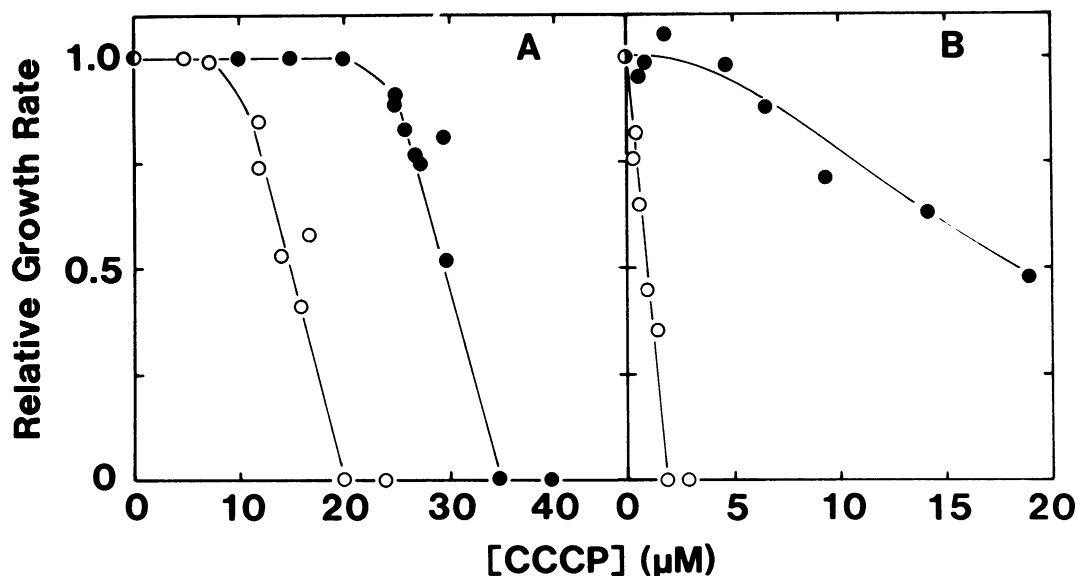

FIG. 2. Growth of *E. coli* ML308-225 and *V. costicola* at pH 6.5 and 8.5 on media containing CCCP. The growth of *E. coli* ML 308-225 (A) and *V. costicola* (B) on media containing various concentrations of CCCP was examined at pH 6.5 (MES-NaOH; ○) and 8.5 (Tricine-NaOH; ●). The addition of CCCP and the determination of growth rates were performed as described in the legend to Fig. 1 and in the text. The relative growth rates were plotted as a function of CCCP concentration. In the absence of CCCP, the growth rates at pH 6.5 and 8.5 were 1.58 and 1.28 generations per h for *E. coli* and 0.56 and 0.52 generation per h for *V. costicola*, respectively.

8.5. These results suggest that the Na^+ extrusion system found in *E. coli* grown on alkaline, Na^+-rich media is a secondary transport system driven by the proton motive force. In contrast to *V. alginolyticus*, the $\Delta\psi$ generated by the Na^+-loaded cells of *E. coli* at both pH 6.0 and 8.5 was completely collapsed by CCCP (data not shown). These observations also indicated that no electrogenic ion pump, other than the H^+ pump, is detectable in *E. coli*.

V. costicola requires a higher concentration of Na^+ for growth than does *V. alginolyticus* (8), and the electron transport activity of membranes isolated from both vibrios, but not from *E. coli*, is dependent on Na^+ (30). Hence, a Na^+ pump was next sought in *V. costicola* by an oxygen-pulse method, which is useful for detecting not only H^+ pumps but also Na^+ pump activity (28). Since the Na^+ pump of *V. alginolyticus* generates $\Delta\psi$ in the presence of CCCP, its action results in the uptake of H^+. Furthermore, when $\Delta\psi$ is collapsed by the permeant cation TPP^+, H^+ uptake in the presence of CCCP is abolished, whereas respiratory H^+ extrusion is markedly stimulated (28). Based on these findings, the anaerobic suspensions of *V. costicola* in the presence of 1.2 M NaCl were pulsed with oxygen at pH 8.5 (Fig. 5). With TPP^+ present, but CCCP absent (Fig. 5A), the oxygen pulse led to a transient acidification of the external medium, which indicates the extrusion of H^+. On the other hand, in the presence of CCCP but without TPP^+ (Fig. 5C), oxygen pulses caused a rapid alkalinization of the medium, which indicates the uptake of H^+. On oxygen exhaustion, the pH of the medium returned to near control levels in both cases. When oxygen was pulsed in the

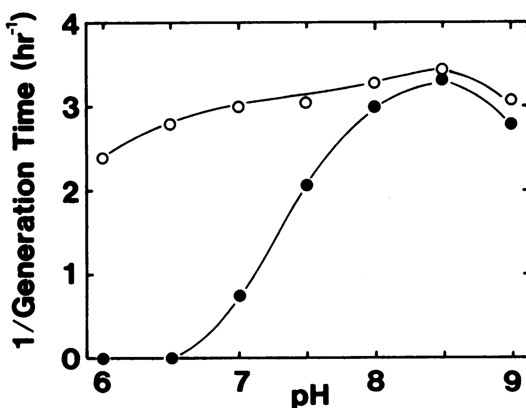

FIG. 3. CCCP-resistant growth and medium pH. The growth rates of *V. alginolyticus* on the complex medium with (●) or without (○) 5 μM CCCP were determined as mentioned in the legend to Fig. 1 over the pH range of 6 to 9. The buffers used at a final concentration of 50 mM were MES (pH 6.0 and 6.5), HEPES (pH 7.0 and 7.5), and Tricine (pH 8.0, 8.5, and 9.0).

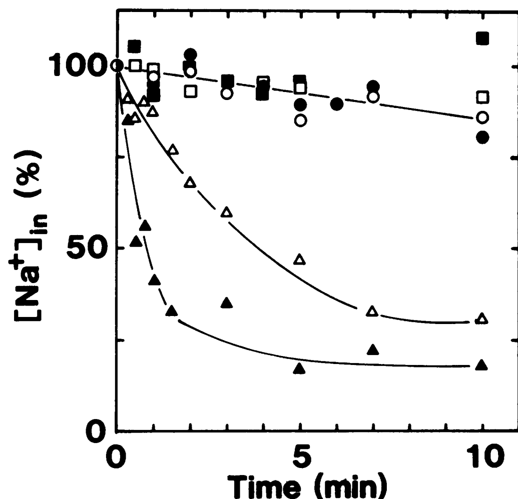

FIG. 4. Na^+ extrusion by *E. coli* ML308-225 grown on an alkaline medium containing NaCl. *E. coli* cells grown on the Tricine-buffered medium (pH 8.5) containing 0.3 M NaCl were harvested at the late-logarithmic phase of growth. Na^+-loaded cells (1.9 mg of protein per ml) equilibrated with $^{22}NaCl$ (7.5 × 10^3 cpm/μl) were prepared at pH 6.0 (open symbols) and 8.5 (closed symbols) as mentioned in the text. Before the start of the experiments, samples (50 μl) of the cell suspensions were transferred to a series of vessels and incubated for 5 min at 25°C in the presence of 10 mM glucose. At 0 time, additions of 10 mM KCl (triangles), 10 mM KCl plus 50 μM CCCP (squares), or nothing (circles) were made. The level of $^{22}Na^+$ retained by the cells was determined at given times by the filtration method and corrected for background radioactivity as described elsewhere (28). The results are given as the percentage of radioactivity at 0 time (about 1.4 × 10^3 cpm).

presence of both CCCP and TPP^+ (Fig. 5B), no change in the medium pH was seen. From the results shown in Fig. 5A and B, it is clear that 10 μM CCCP makes membranes permeable to H^+ and inhibits the net extrusion of H^+. Furthermore, from the results shown in Fig. 5B and C, it is also evident that the uptake of H^+ observed in the presence of CCCP is driven by $\Delta\psi$. When the assays were performed in 1.2 M LiCl instead of NaCl, the extrusion of H^+ did take place in the absence of CCCP (Fig. 5D), but not in its presence (Fig. 5E). These results indicate that *V. costicola* possesses a respiration-dependent electrogenic ion pump specific to Na^+, in addition to the H^+ extrusion system. Since H^+ uptake in the presence of CCCP was not observed at pH 6.5 and since CCCP-resistant $\Delta\psi$ was generated at pH 8.5, but not at pH 6.5, the Na^+-specific pump also has a pH optimum in the alkaline region. Note that oxygen-pulse assays performed with Na^+-loaded *E. coli* did not show any H^+ uptake in the presence of CCCP at all pH values tested (data not shown).

Effect of growth media and salts on ΔpH and $\Delta\psi$ of *V. alginolyticus*. As reported elsewhere (27, 28) and shown in Table 1, Na^+-loaded cells of *V. alginolyticus* in a buffer containing NaCl as the sole salt generates a $\Delta\psi$ that is Na^+ pump dependent and CCCP resistant at pH 8.5. Under these conditions, the intracellular pH was near 6, and the ΔpH (in millivolts) was about equal to the $\Delta\psi$. The maintenance of the intracellular pH near neutrality is considered to be essential for bacterial growth (for a review, see reference 23) and, indeed, the intracellular pH of *V. alginolyticus* is maintained at about 8 (25). Therefore, it was of interest to examine whether or not the intracellular pH of cells growing in a CCCP-containing medium was still maintained at a pH of about 8. Both the intracellular pH and $\Delta\psi$

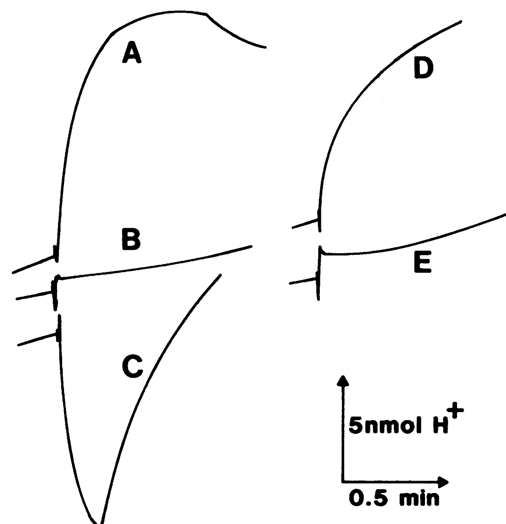

FIG. 5. H^+ extrusion and Na^+ pump-dependent H^+ uptake by *V. costicola*. Harvested cells of *V. costicola* were washed twice with and suspended in 1.2 M NaCl and 10 mM $MgCl_2$ to give a final concentration of 45 mg of protein per ml. Samples (40 μl) of the cell suspensions were added to 2 ml of 1.2 M NaCl (A to C) or LiCl (D and E) containing 10 mM $MgCl_2$ and 0.2 mM Tricine-NaOH, pH 8.5. CCCP (B, C, and E) and TPP^+ (A, B, and D) were also included in the reaction mixture at final concentrations of 10 μM and 1 mM, respectively. The cell suspensions were kept anaerobic under the stream of nitrogen at 25°C until the medium pH became near constant. After the readjustment of the pH to 8.5, an oxygen pulse was made by the addition of 100 μl of air-saturated 1.2 M NaCl (A to C) or LiCl (D and E). Changes in the medium pH were monitored by pH electrode as described in the text. An upward change indicates acidification. The spike observed at the addition of salt solution is the response of the electrode to the insertion and removal of the pipette.

TABLE 1. Effects of salts on $\Delta\psi$ and intracellular pH of *V. alginolyticus* at pH 8.5[a]

Medium	CCCP (20 μM)	$\Delta\psi$ (mV)	pH_{in}	$-59\Delta pH$ (mV)	Δp (mV)
Complex[b]	−	−137	8.29	12	−125
	+	−59	8.29	12	−47
Synthetic[b]	−	−126	7.84	39	−87
	+	−56	7.98	31	−25
NaCl[c] (0.4M)	−	−154	8.10	24	−130
	+	−156	6.29	130	−26
NaCl (0.4M)	−	−135	7.97	31	−104
plus KCl[c] (14 mM)	+	−87	8.05	27	−60

[a] The assays were performed as described in the text. Intracellular pH and $\Delta\psi$ in the presence and absence of 20 μM CCCP were determined in the respective assay medium containing 50 mM Tricine-NaOH (pH 8.5) and 0.1 mg of chloramphenicol per ml.

[b] The cells were grown on, washed with, and assayed in the specified growth medium.

[c] Na^+-loaded cells were prepared from cells grown on the synthetic medium and assayed in the specified salt solution.

were determined in *V. alginolyticus* at pH 8.5 incubated in various media (Table 1). Although CCCP significantly collapsed Δp under all of the conditions tested, the effects of CCCP on intracellular pH and $\Delta\psi$ varied depending on the conditions. In growth media, the intracellular pH was maintained at a pH of about 8 in the presence of CCCP, whereas the $\Delta\psi$ was collapsed by CCCP. In contrast, in the Tricine-buffered NaCl medium containing 0.4 M NaCl, the $\Delta\psi$ was not affected by CCCP, but the interior of the cells was acidified to pH 6.29. When the concentration of K^+ included in the Tricine-NaCl buffer was the same as that in the synthetic medium (14 mM), CCCP again dissipated the $\Delta\psi$ and had no effect on the intracellular pH, similar to the effect seen in the growth media.

The intracellular concentrations of K^+ and Na^+ were determined in *V. alginolyticus* growing on the synthetic medium containing 14 mM K^+ and 0.3 M Na^+. The addition of 5 μM CCCP during the logarithmic phase of growth immediately terminated the growth at pH 6.5 and caused a rapid leakage of K^+ from cells. On the other hand, CCCP added at pH 8.5 did not prevent growth; the intracellular concentrations of K^+ (about 0.4 M) and Na^+ (about 60 mM) were essentially the same as those determined in the absence of CCCP. Hence, the chemical potential of Na^+ under these conditions is about −40 mV. The sum of this and $\Delta\psi$ (−56 mV) indicates that a Na^+ electrochemical potential of substantial magnitude (about −100 mV) is still generated in the presence of CCCP.

DISCUSSION

The growth of *V. alginolyticus* and *V. costicola* was resistant to CCCP; the inhibition was not rendered less effective in alkaline growth media, since CCCP inhibited the growth of *E. coli* on similar alkaline medium and, moreover, membranes of the organisms under all of the conditions tested were made permeable to H^+ by an appropriate concentration of CCCP. Thus, the Na^+ pump found in both *V. alginolyticus* and *V. costicola* is most likely to be responsible for the mechanism of CCCP-resistant growth. *E. coli*, in which no Na^+ pump was found, showed CCCP-sensitive growth under all of the conditions tested. Moreover, the growth of *V. alginolyticus* and *V. costicola* became sensitive to CCCP under conditions where the Na^+ pump had little activity. Furthermore, the isolation of CCCP-sensitive mutants of *V. alginolyticus* that are defective in the Na^+ pump (24a) gives further support to this conclusion.

The occurrence of CCCP-resistant mutants of *Bacillus megaterium* (2, 3, 9, 10) and *E. coli* (14, 15) has been reported. These mutants are altered in the proton-translocating ATPase. In contrast, in *V. alginolyticus*, the synthesis of ATP in the presence of CCCP is performed by substrate-level phosphorylation (28), making it unlikely that the ATPase is involved in the CCCP-resistant growth of *V. alginolyticus*.

The fact that *V. costicola*, which is likely to be of marine origin (1), also possesses the respiration-dependent Na^+ pump raises an intriguing possibility that such a Na^+ pump may be widely distributed among halophilic, especially marine, bacteria. As an exceptional mechanism, however, the Na^+ pump may also exist in nonhalophilic bacteria. Indeed, Dimroth (4–6) has discovered a Na^+ pump driven by the decarboxylation of oxaloacetate in *Klebsiella aerogenes*, and Heefner and Harold (13) have reported an ATP-driven Na^+ pump in *Streptococcus faecalis*. In this connection, CCCP-resistant growth may be an excellent method to detect bacteria having a Na^+ pump.

Although the roles of the cytoplasmic cations in cellular metabolism are not completely understood (7, 12, 18), their significance is clearly demonstrated by colicins E1, Ia, and K, which make membranes permeable to various ions (for a review, see reference 16). The rescue of colicin K-treated cells by the supply of excess K^+ and Mg^{2+} (17) suggests that the maintenance of a cytoplasmic cation environment is essential for survival. Similarly, the growth of *S. faecalis* in the presence of gramicidin, which makes membranes permeable to K^+, Na^+, and H^+, requires a rich medium, an alkaline pH, and a high concentration of K^+ (11). In both cases, a medium similar to the cytoplasm is necessary for

growth under otherwise lethal conditions. Consistent with this, the inhibition of H^+ circulation at acidic pH by CCCP is lethal to *V. alginolyticus*, since only the proton motive force is the energy transducer at acidic pH. On the other hand, when the Na^+ pump is functional, *V. alginolyticus* was able to maintain the cytoplasmic cation environment in the presence of CCCP. Since the Na^+ extrusion is the direct result of the Na^+ pump and the accumulation of K^+ is tightly coupled to Na^+ extrusion (26, 27), the intracellular concentrations of Na^+ and K^+ can be maintained at nearly normal levels in the presence of CCCP or in the absence of H^+ circulation. The maintenance of the cytoplasmic pH in the presence of CCCP also requires K^+, as has been observed in the absence of CCCP (25). It is likely that the electrogenic influx of K^+ prevents the extensive acidification of cytoplasm by collapsing the Na^+ pump-generated $\Delta\psi$ and thus preventing excessive H^+ influx. Moreover, when necessary, acidification through a K^+/H^+ antiport (22), driven by the concentration gradient of K^+, may participate in the maintenance of the intracellular pH. From these considerations, it is reasonable to conclude that the reason for CCCP-resistant growth in these cells is the maintenance of cationic environments by the Na^+ pump. It also seems likely that an electrochemical potential of Na^+ helps the cells to grow in the presence of CCCP. Finally, we emphasize that the data presented in this paper show that the growth of bacteria that have a Na^+ pump is not governed by H^+ circulation.

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