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Proton Circulation in *Vibrio costicola*†

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The importance of proton movements was assessed in the moderate halophile *Vibrio costicola*. When anaerobic cells in acidic buffer (pH 6.5) were given an O_2 pulse, protons were extruded regardless of the presence of Na^+. At pH 8.5, however, *V. costicola* produced an acidic response to an O_2 pulse in the absence of Na^+ and an alkaline response when Na^+ was present. An Na^+/H^+ antiport activity was confirmed at pH 8.5. All of these effects were prevented by protonophores or butanol treatment. Growth in complex medium at pH 8.5 was prevented by a high concentration (50 μM) of carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) or a low concentration (5 μM) of another protonophore, 3,3',4',5-tetrachlorosalicylanilide (TCS). The relative ineffectiveness of the former protonophore was caused by the proteose peptone and tryptone ingredients of the complex medium, since 5 μM completely prevented growth in their absence. The results are explained by a primary respiratory-linked proton efflux coupled to a secondary Na^+/H^+ antiport operating at alkaline pH. Evidence was seen for a role of Na^+ in stimulating proton influx at alkaline pH, presumably via the pH homeostasis mechanism.

Vibrio costicola is a moderate halophile capable of growing optimally over the pH range 6.5 to 9.0 in the presence of 1.0 M NaCl (8). This organism is a good model, therefore, for comparing the energetics of ion movements in relation to Na^+-dependent growth at acidic and alkaline pH values.

Several functions for Na^+ have been identified in *V. costicola*. Although cell lysis may be prevented by salts other than NaCl, functions which are Na^+ specific include active transport of α-aminoisobutyric acid (AIB), Na^+/H^+ antiport activity, and formation and maintenance of the membrane potential ($\Delta\psi$). In the transport of AIB, Na^+ lowers the K_m at least 10-fold (manuscript in preparation) and is presumed to cotransport with AIB to explain the close correlation found between transport and the magnitude of $\Delta\psi$ (9). AIB transport in the alkalophile *Bacillus alkalophilus* similarly shows an Na^+ requirement and dependence on $\Delta\psi$ (7). In *V. costicola*, an Na^+/H^+ antiporter was identified at pH 7.5, and Na^+ was implicated in the pH homeostasis mechanism when the cells were in alkaline media (8).

One of the most puzzling functions to explain is the need for Na^+ to form, and maintain, $\Delta\psi$ (9). Two different mechanisms have been suggested. The first, applied to *V. costicola*, links the primary efflux of protons during respiration at alkaline pH to a secondary electrogenic Na^+/H^+ antiporter (8). The resulting development of a $\Delta\psi$ (inside negative) and a sodiummotive force, as well as pH homeostasis, can be explained by the stoichiometry of the reactions. This mechanism is consistent with data presented for alkalophilic bacteria (6, 14, 15) and *Escherichia coli* (2, 22). The second mechanism, presented initially in detail for *Vibrio alginolyticus* (19) and later extended to *V. costicola* (20), involves a change of the respiratory chain from a proton pump at acidic pH to a direct Na^+ pump at alkaline pH. The energetics of motility in *V. alginolyticus* (3) and of Na^+ movements in the halotolerant bacterium Ba_1 (13) have been interpreted according to this concept.

A distinctive feature of the primary respiratory-linked Na^+ pump, as previously hypothesized (19, 20), is protonophore-insensitive growth in alkaline media. Here, we present evidence that the growth of *V. costicola* is protonophore sensitive in a defined alkaline medium. Previously, we have shown that transport of AIB and $\Delta\psi$ is sensitive to protonophores at pH 8.7 (9). Further in accord with a model in which proton efflux is the primary transport mechanism, we show that a net influx of protons occurs at alkaline pH, and we measure a protonophore-sensitive Na^+/H^+ antiport activity.

MATERIALS AND METHODS

Bacterial growth. *V. costicola* NRCC 37001 (National Research Council, Ottawa, Ontario, Canada) was grown at 30°C with vigorous shaking in 500-ml Erlenmeyer flasks containing 100 ml of medium. The complex growth medium included 1% (wt/vol) proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.) and 1% (wt/vol) tryptone (Difco) (referred to as PPT in the text) plus 1.0 M NaCl and 50 mM tricine [*N*-tris (hydroxymethyl)methylglycine]-NaOH (final pH of 8.5). For some growth experiments, a synthetic medium was used (5), modified to contain 1 M NaCl, 72 mM K_2HPO_4, 8 mM KH_2PO_4, 0.4 mM $MgSO_4$, 37 mM NH_4Cl, 15 mM $(NH_4)_2SO_4$, 20 mM glucose, and 50 mM tricine-NaOH (final pH of 8.5). In some vials, 1% (wt/vol) PPT was also added. These synthetic media were sterilized by filtration with 0.2-μm Nalgene filters (Nalge/Sybron Corp., Rochester, N.Y.).

Growth was monitored hourly by measurements of optical density at 660 nm (1-cm light patch) and pH tested with an Orion digital pH meter. Cultures were maintained as previously described in complex medium (8).

Effect of external pH on respiration-dependent flux of protons. At the end of the exponential phase of growth in complex medium (16 to 18 h, 1.0 to 1.5 mg [dry weight] per ml), bacteria were washed twice with a saline solution containing 1.0 M NaCl, 8 mM KCl, 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, 50 mM sodium thiocyanate, and 1 mM Tris-hydrochloride (final pH of 8.5) or 1 mM Mes [2-(*N*-morpho-

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lino)ethanesulfonic acid]-NaOH (final pH of 6.5). The bacterial suspension (3 to 4 mg [dry weight] per ml) was stored on ice before being used, usually for less than 4 h.

Five milliliters of bacterial suspension was transferred into the reaction vessel as described above, and the pH was adjusted to 6.5 or 8.5 by adding small amounts of HCl or NaOH (1 N). Solutions of 4 M NaCl were prepared in water and saturated with either O_2-free N_2 or O_2 in closed 5-ml serum bottles, and 50 μl was added to the anaerobic suspension. The uncouplers carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3,3′,4′,5-tetrachlorosalicylanilide (TCS) and controls with butanol-treated cells were included.

Proton efflux and Na^+/H^+ antiport activities at pH 8.5. At the end of the exponential phase of growth in complex medium, bacteria were washed twice with an Na^+-free solution containing 1.0 M KCl, 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, 50 mM potassium thiocyanate, and 1 mM Tris-hydrochloride (final pH of 8.5). The bacterial suspension (3 to 4 mg [dry weight] per ml) was stored in the Na^+-free solution on ice before being used, usually for less than 4 h.

Five milliliters of bacterial suspension was transferred into the reaction vessel with 100 μg of carbonic anhydrase and kept anaerobic under a continuous N_2 flow, as previously described (8). The pH was adjusted to 8.5 by adding small amounts of HCl or KOH (1 M).

Solutions of 4 M NaCl or 4 M KCl were prepared in water and saturated with O_2-free N_2 or with O_2 in closed 5-ml serum bottles. When required, 50-μl samples were added to the anaerobic bacteria.

Effects of the uncouplers CCCP (20 μM) and TCS (20 μM) and controls with butanol-treated cells were tested as previously described (8).

Inhibitors. The ionophores CCCP (Sigma Chemical Co., St. Louis, Mo.) and TCS (Fisher Scientific Co., Nepean, Ontario, Canada) were prepared as methanolic solutions; appropriate controls were run to account for any effects of methanol alone.

Dry weight. The dry weight of bacteria was determined as previously described (8) by 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. To determine the dry weight of the inoculum in the growth experiments, bacteria were first washed in a saline solution containing 1 M NaCl, 8 mM KCl, 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, and 50 mM Tris-hydrochloride (pH 7.2).

RESULTS

Effect of external pH on respiration-dependent flux of protons. Respiration provides the energy needed by *V. costicola* for the expulsion of protons at pH 7.5 (8). Since respiration rate is optimal at pH 8.2 to 8.7 (9), as in *V. alginolyticus* (19) and pH regulation requires a return of protons to the cytoplasm at alkaline pH (>7.5), we chose to study the effect of pH on respiration-dependent proton movements.

Proton efflux, as induced by an O_2 pulse, was compared in cells incubated in 1.0 M NaCl at pH 6.5 and 8.5. An acidification of the medium occurred in response to an O_2 pulse given to anaerobic cells at pH 6.5 (Fig. 1A). This efflux of protons was prevented by incubation of the bacteria with CCCP or TCS. During these studies, the protonophores were added as methanolic solutions, because methanol (unlike certain other alcohols) is not oxidized by these cells (9). An equivalent amount of methanol did not affect the results obtained. Also shown are controls in which acidification did not occur with butanol-treated cells or after injections of O_2-free solutions.

In contrast to the above response, at pH 8.5 an O_2 pulse given to the anaerobic bacterial suspension resulted in an alkalinization of the medium (Fig. 1B). This proton movement was prevented by either CCCP or TCS. Again, no net proton movement was detected when an O_2 pulse was given to butanol-treated cells or when N_2-saturated solutions were used. Also, methanol itself caused no inhibition of the alkaline response.

Proton efflux and Na^+/H^+ antiport activities at pH 8.5. In view of the alkalinization of the medium in response to an O_2 pulse at pH 8.5, it is important to demonstrate that respiratory-linked proton efflux does occur at alkaline pH. Although this can readily be demonstrated at pH 7.5, at which there is no pH gradient across the cytoplasmic membrane (8), it is necessary to remove exogenous Na^+ to measure net proton efflux at higher pH values. The cells require protection against lysis on removing NaCl, which is best done with retention of metabolic activities by substituting with 1 M KCl (9). This is shown in Fig. 2A, where a rapid acidification of the medium occurred in response to an O_2 pulse given to anaerobic cells in a 1 M KCl solution. As the O_2 was consumed, the external pH returned close to its original value, as expected from a resorption of protons by the bacteria. The acidification of the medium in response to an O_2 pulse was prevented by the protonophores CCCP and TCS, added as methanolic solutions; an equivalent amount of methanol did not significantly affect the acidification observed. No acidification occurred when an O_2 pulse was injected into butanol-treated bacteria or when an N_2-saturated solution was injected into the anaerobic cell suspensions.

The Na^+/H^+ antiporter previously identified at pH 7.5 (8) was also found to be active at alkaline pH, as shown by the acidification of the external medium when an anaerobic solution of NaCl was injected into cells suspended in O_2-free 1.0 M KCl solution (Fig. 2B). This activity at pH 8.5 was also prevented by the uncouplers CCCP and TCS (dissolved in methanol). Methanol alone caused only a small inhibition of activity. After the initial burst of proton efflux (Fig. 2B, curves a and b), a much larger resorption of protons was detected. No acidification occurred when an O_2 pulse was given to butanol-treated cells or when N_2-saturated 4 M KCl solution was injected. Although not illustrated in the figure, no inhibition of proton efflux was caused by the diuretic amiloride, often effective against eucaryotic Na^+/H^+ antiport activity (15).

Effect of uncouplers on bacterial growth at alkaline pH. *V. costicola* can grow in a complex medium at alkaline pH, as previously reported (8), with a generation time of 1.36 h. The presence of 5 μM CCCP in the culture medium had no effect on growth, although 20 μM CCCP produced a slightly longer generation time (1.80 h), and a concentration of 50 μM CCCP completely inhibited growth (Fig. 3). However, at all concentrations tested (5 to 50 μM), TCS had a complete inhibitory effect on growth. Since the protonophores were added as methanolic solutions, we also showed that the amount of methanol used (up to 25 mM) did not influence bacterial growth (curve for cells lacking methanol is not shown).

When bacteria were grown at alkaline pH on synthetic medium, no growth at all was detected in the presence of 5 μM CCCP, and 1% PPT relieved the inhibition by CCCP (Fig. 4). This has particular relevance since the growth

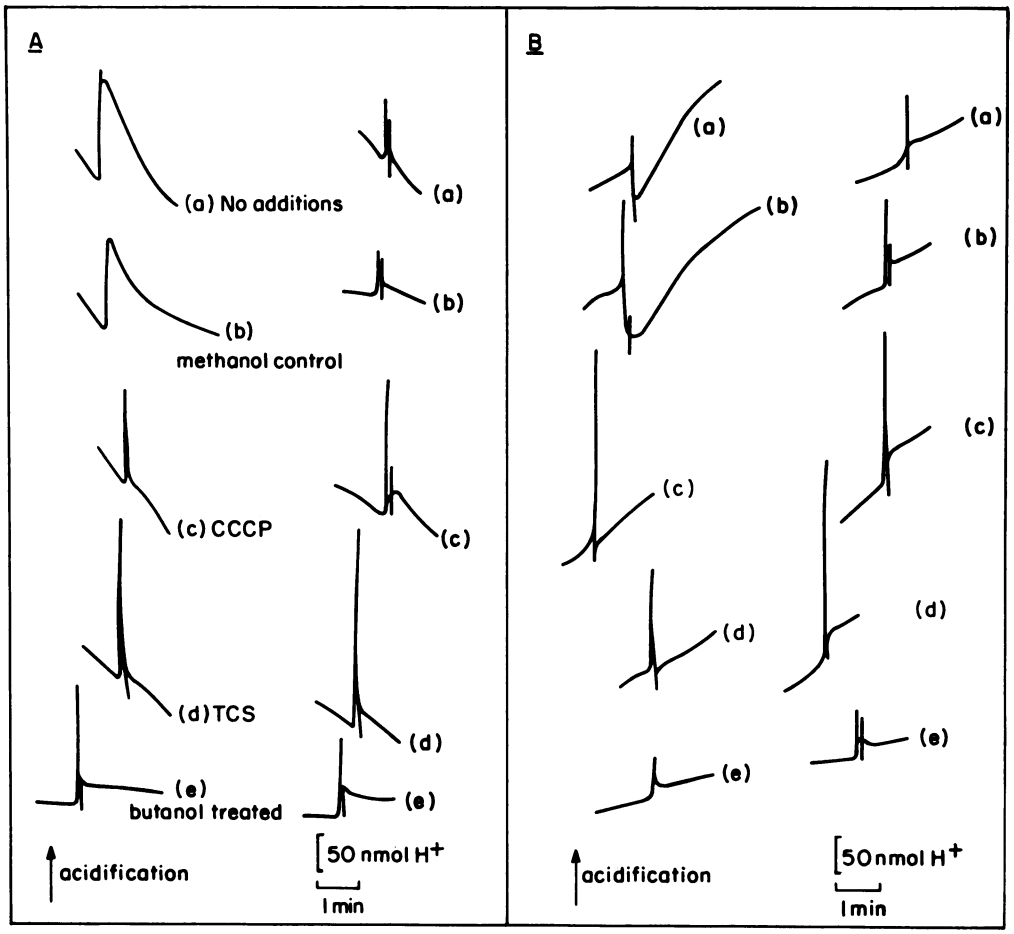

FIG. 1. Influence of external pH on respiration-dependent proton flux. Bacteria were incubated in 1 M NaCl at pH 6.5 (A) or 8.5 (B). Proton movement was measured with a pH electrode as a change in external pH resulting from injections of O_2-saturated 4 M NaCl solution to anaerobic cell suspensions (curves a to e on the left of A and B). The cells received injections of O_2-free, N_2-saturated 4 M NaCl solutions (curves a to e on the right of A and B). Additions made before O_2- or N_2-saturated 4 M NaCl were: a, no additions; b, 47 mM methanol; c, 20 μM CCCP; d, 20 μM TCS; and e, no additions, butanol-treated cells.

experiments of Tokuda and Unemoto with *V. costicola* were conducted in a complex medium (20).

DISCUSSION

The model proposed to describe the energetics of ion movements in *V. costicola* is one in which respiration causes an efflux of protons as the primary transport event. The resulting protonmotive force is then coupled to an electrogenic Na^+/H^+ antiporter to develop a sodiummotive force and aid in pH homeostasis (8, 9). The sodiummotive force appears to play a role in the Na^+-dependent transport of AIB (9). Since other workers have concluded that the need for proton circulation during growth of *V. costicola* at alkaline pH can be replaced by a direct Na^+ pumping during respiration (20), the evidence for the above model was reassessed.

The influence of Na^+ and pH on the steady-state rate of proton translocation is dramatic. When exogenous Na^+ is absent, *V. costicola* ejects protons to the medium in response to an O_2 pulse at both pH 6.5 and 8.5 (8; Fig. 2). The addition of Na^+ followed by a pulse of O_2 to the anaerobic cell suspension at pH 6.5 caused a decrease in the net efflux of protons, through Na^+/H^+ antiport activity (8; this study). At pH 8.5, however, the presence of cytoplasmic Na^+ has the much more dramatic effect of reversing net proton movement to an inward direction (Fig. 1). These results are in accord with the prediction that Na^+/H^+ antiport activity should be most rapid at alkaline pH to maintain a pH gradient of orientation acidic inside. Recently, a similar alkalinization of the medium was found in response to the addition of catalytic amounts of Na^+ to the halotolerant bacterium Ba_1 at alkaline pH (13). A stoichiometry of proton efflux during respiration to proton influx and Na^+ efflux through the antiporter could be 2:3:2, as predicted by Krulwich (15) for alkalophilic bacilli. This allows a net translocation of one proton inward formation of $\Delta\psi$ (negative inside) and formation of a sodiummotive force. Our results showing that protonophores prevented the alkalinization of the medium when *V. costicola* received an O_2 pulse (Fig. 1) disagree with those of Tokuda and Unemoto (20). Although they did not report the effect of an O_2 pulse on net proton movement in the absence of CCCP, in the presence of the protonophore protons entered their cells. Whether the alkalinization would have been much larger in the absence of CCCP in their strain of *V. costicola* is unknown.

V. costicola grows optimally from pH 6.5 to 9.0 (8). As the medium is adjusted to more alkaline values, the respiratory rate and the magnitude of the membrane potential increase greatly, but the protonmotive force declines because of the inversion of the pH gradient to acidic inside at about pH 7.5

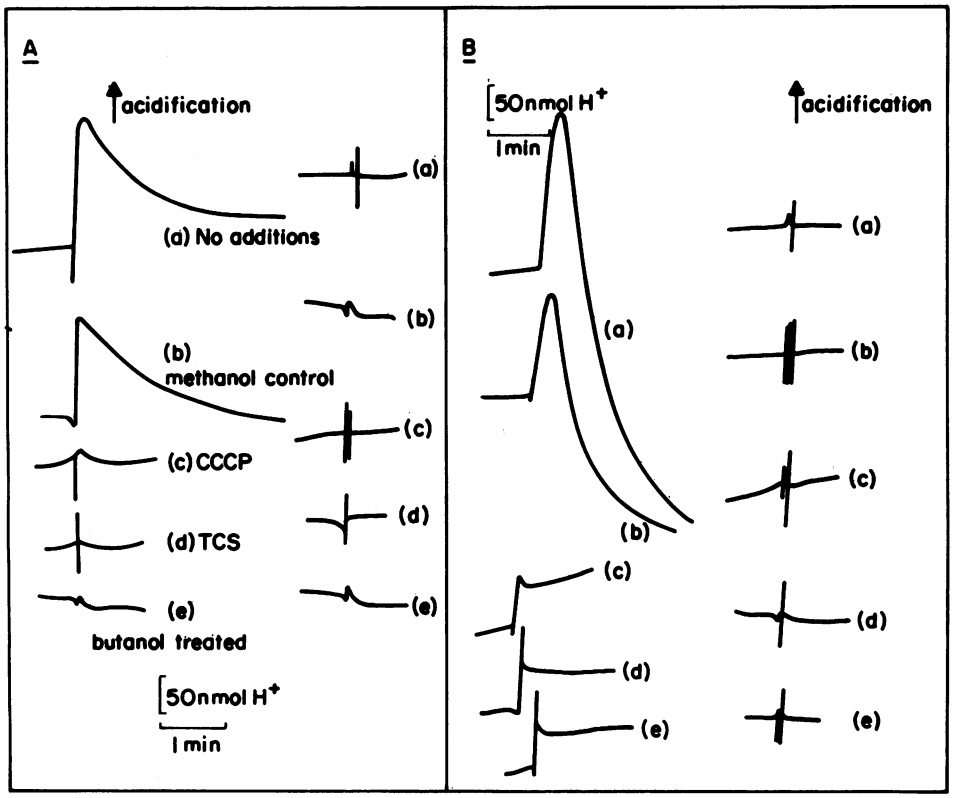

FIG. 2. Respiration-linked proton efflux and Na^+/H^+ antiport at pH 8.5. (A) Respiration-linked proton efflux. Proton efflux was measured with a pH electrode as the increase in acidity resulting from injections of O_2-saturated 4 M KCl solution (40 mM final concentration) to anaerobic cells suspended in 1 M KCl. Anaerobic bacteria were preincubated for 20 min at 30°C with inhibitors before the O_2 pulse. Additions were: a, no additions; b, 47 mM methanol; c, 20 μM CCCP; d, 20 μM TCS; and e, no additions, butanol-treated cells. A similar series of experiments (curves a to e on the right) was performed in which the anaerobic bacteria received injections of an O_2-free, N_2-saturated 4 M KCl solution. (B) Evidence for an Na^+/H^+ antiport activity. Proton efflux was measured with a pH electrode as the increase in acidity resulting from injections of 50 μl of 4 M anaerobic NaCl solution (40 mM final concentration) to anaerobic cells suspended in 1 M KCl. Anaerobic bacteria (curves b to e on the left) were preincubated with the reagents as for A; the cells received injections of O_2-free, N_2-saturated 4 M KCl solution (curves a to e on the right).

(8, 9). In the absence of exogenous Na^+, $\Delta\psi$ collapses, reforming completely upon the addition of Na^+ to respiring cells (9). Exogenously added Na^+ is not needed for respiration (9), although Na^+ has been previously shown to activate NADH oxidase in membrane preparations of another strain of *V. costicola* (21). Evidently, sufficient cytoplasmic Na^+ was present during our studies for respiration and ATP synthesis to continue but not for $\Delta\psi$ to be maintained (9). The importance of these findings is in separating the requirements for Na^+ to establish and maintain $\Delta\psi$ from any need for smaller amounts of Na^+ to activate respiratory enzymes. Thus, in *V. costicola* a recovery of $\Delta\psi$ in response to additions of Na^+ does not provide evidence to favor respiratory activity as a direct Na^+ pump. A key question is, if proton efflux (and not that of Na^+) is the primary transport event, then why is Na^+ required to form and maintain $\Delta\psi$ in cells suspended in 1 M KCl at pH 8.5? These conditions of Na^+ depletion lead to a rapid efflux of protons when an O_2 pulse is given (Fig. 2A), resulting in an expected rise in the cytoplasmic pH in the absence of Na^+/H^+ antiport activity. A precipitous inhibition in respiration observed at pH values greater than 9.0 (9) would cause a decrease in proton efflux. An inhibition of respiration in response to a rise in the cytoplasmic pH in Na^+-deficient cells would slow proton efflux and thus provide some control to prevent the cytoplasm from becoming excessively alkaline. In this case, respiration would be dependent on Na^+, not through the direct activation of respiratory enzymes, but rather as a consequence of the loss of pH regulation.

For regulation of the cytoplasmic pH with Na^+, not only is an Na^+/H^+ antiporter required but also a means to replenish cytoplasmic Na^+ (1, 4, 10, 16). Recent evidence suggests that in a facultative alkalophile the rate of entry of Na^+ may be regulated by the cytoplasmic pH (17). The injection of Na^+ to Na^+-deficient *V. costicola* gave an initial acidification of the medium, followed by a much larger alkalinization (Fig. 2B). Two explanations which have yet to be tested seem possible. In the first, the initial influx of Na^+ during Na^+/H^+ antiport could result in an activation by cytoplasmic Na^+ of K^+/H^+ antiport, for example. Secondly, the addition of exogenous Na^+ with initial antiport activity would cause the cytoplasmic pH to become more alkaline. This change in the cytoplasmic pH may allow more Na^+ to enter the cells through part of the Na^+ cycle (1), with a resulting reversal of Na^+/H^+ antiport to the physiological direction. The energy for this process could come from $\Delta\psi$ (inside negative) established by the initial antiport of two Na^+ inside to three H^+ outside (see above).

The effectiveness of the protonophores CCCP and TCS in preventing growth at pH 8.5 illustrates the importance of proton flux in *V. costicola*. Both protonophores at 20 μM concentration inhibit AIB transport by ca. 98%, cause a

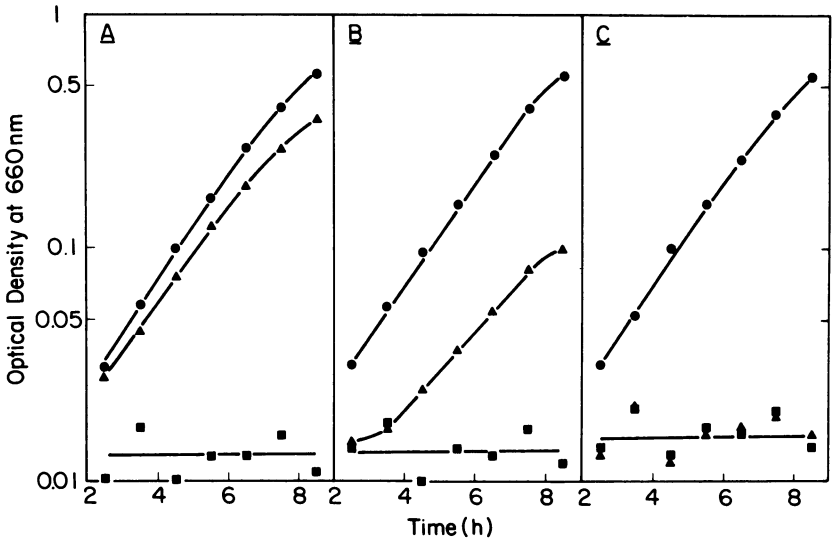

FIG. 3. Influence of uncouplers on growth of *V. costicola* in complex medium at pH 8.5. *V. costicola* was grown on complex medium containing 1% PPT, 1 M NaCl, and 50 mM tricine-NaOH (pH 8.5) in the presence of the uncouplers CCCP (▲) or TCS (■), or methanol (control) (●). The final concentrations of the uncouplers were 5 (A), 20 (B), and 50 (C) μM with, respectively, 2.5, 10, and 25 mM methanol. The inoculum consisted of 0.2 mg (dry weight) of an exponential-phase culture, grown overnight in the same complex medium. Growth was monitored hourly by measurements of the optical density at 660 nm.

dramatic decline in $\Delta\psi$, and inhibit Na^+/H^+ antiport activity, showing that these reagents can function at the alkaline incubation conditions. The relative ineffectiveness of CCCP observed by Tokuda and Unemoto (20) at pH 8.5 compared with pH 6.5 may relate to the use of a complex growth medium and perhaps to an expected higher potency of the protonophore at more acidic pH (12), rather than indicating a direct Na^+ pumping by the respiratory chain of *V. costicola*. When CCCP is added to a complex medium containing the inoculum (20), a competition is expected between the distribution of CCCP into the cell membrane and chemical reaction with aminothiols, such as cysteine, present in the medium. The effect of pH on these distributions is not known. Since CCCP is known to react rapidly with aminothiol compounds, with 1 mM cysteine protecting against the uncoupling action of CCCP in mitochondria (11) and methanogenic bacteria (12), the effect of adding complex medium ingredients on the inhibition of growth by CCCP was studied. Indeed, proteose peptone (1% [wt/vol]) and tryptone (1% [wt/vol]) prevented the growth inhibition, showing the importance of using a defined medium for studies involving CCCP (Fig. 3 and 4).

There are clearly many differences in the energetics of *V. alginolyticus* and *V. costicola*, particularly with respect to

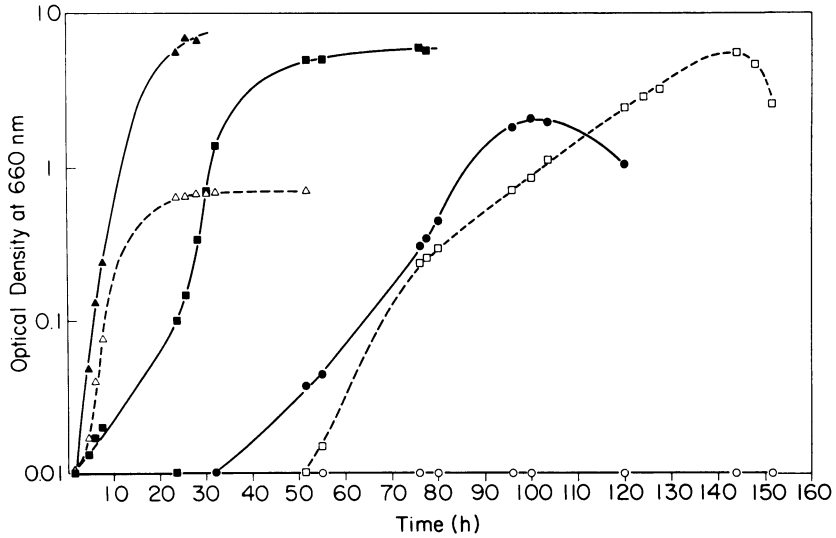

FIG. 4. Influence of medium composition on the effectiveness of CCCP as an inhibitor of the growth of *V. costicola*. *V. costicola* was grown with 1 M NaCl and 50 mM tricine-NaOH (pH 8.5) in complex medium containing 1% PPT (▲, △), in synthetic medium (●, ○), or in synthetic medium plus 1% PPT (■, □). A methanolic solution of CCCP (5 μM final concentration (△, ○, □) or of methanol as a control (2.5 mM final concentration) (▲, ●, ■) was then added. The inoculum consisted of 0.2 mg (dry weight) of an exponential-phase liquid culture grown in the synthetic medium alone. Growth was monitored by measurements of the optical density at 660 nm.

the different sensitivities at alkaline pH of $\Delta\psi$ and AIB transport to CCCP (8, 9, 18–20). At least for our strain of *V. costicola*, there is presently no strong evidence for a respiratory-linked direct Na^+ pump. Rather, a respiratory-linked primary proton efflux in combination with an electrogenic Na^+/H^+ antiporter can explain growth at alkaline pH.

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