APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1987, p. 2124–2128
0099-2240/87/092124-05$02.00/0

Vol. 53, No. 9

Membrane ATPases and Acid Tolerance of *Actinomyces viscosus* and *Lactobacillus casei*

GARY R. BENDER AND ROBERT E. MARQUIS*

Department of Microbiology and Immunology and Caries Research Center, The University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 23 April 1987/Accepted 24 June 1987

***Lactobacillus casei* ATCC 4646 and *Actinomyces viscosus* OMZ105E were found to differ markedly in acid tolerance. For example, pH profiles for glycolysis of intact cells in dense suspensions indicated that glycolysis by *L. casei* had an optimal pH of about 6.0 and that glucose degradation was reduced by 50% at a pH of 4.2. Comparable values for *A. viscosus* cells were at pHs of about 7.0 and 5.6. The difference in acid tolerance appeared to depend mainly on membrane physiology, and the addition of 40 μM gramicidin to cell suspensions increased the sensitivity of the glycolytic system by as much as 1.5 pH units for *L. casei* and up to 0.5 pH unit for *A. viscosus*. *L. casei* cells were inherently somewhat more resistant to severe acid damage than were *A. viscosus* cells, in that Mg release from *L. casei* cells in medium with a pH of 3.0 occurred only after a lag of some 4 h, compared with rapid release from *A. viscosus* cells. However, the major differences pertinent to the physiology of the organisms appeared to be related to proton-translocating ATPases. Isolated membranes of *L. casei* had about 3.29 U of ATPase per mg of protein, compared with only about 0.06 U per mg of protein for those of *A. viscosus*. Moreover, the ATPase of *L. casei* had a pH optimum for hydrolytic activity of about 5, compared with an optimal pH of about 7 for that of *A. viscosus*. Permeabilities of intact cells to exogenously added protons were found to be minimal at a pH of about 4 for *L. casei* and at a pH of about 6 for *A. viscosus*. The ATPases of both organisms were relatively resistant to inhibition by fluoride, but fluoride did act to lower acid tolerance of intact cells. Overall, it appeared that differences in acid tolerance between the bacteria depend both on the relative amounts of ATPases in the cell membranes and on their biochemical characteristics.**

Dental plaque contains a diverse community of microorganisms living in crowded conditions and subjected to a variety of ecological stresses. There is constant mechanical removal of part of the community due to salivary flow and more severe disruption due to mastication, tooth brushing, flossing, and other intermittent assaults. Food supply may be highly variable. There is now evidence that saliva alone can serve as a growth medium for *Actinomyces viscosus* and for some plaque streptococci (4). However, the diet of the host provides needed nutrients for many or most plaque bacteria, and carbohydrates appear to be growth-limiting factors in saliva cultures of organisms such as *Streptococcus mutans*. Especially in supragingival plaque, the bacteria are also subjected to cycles of acidification, followed by a rise in pH. Plaque pH may drop to 4.0 or slightly lower after exposure to sugars (15). Subsequently, the pH rises because of acid diffusion, buffering by salivary and tooth components, and ammonia production from amino acids, such as arginine (9).

Plaque organisms vary widely in acid tolerance (6). For example, we have found that the minimal pH for glycolysis by the OMZ105E strain of *A. viscosus* is generally above 5.0. The minimal pH for glycolysis by *S. mutans* GS-5 is approximately 4.0, and the minimum pH for glycolysis by *Lactobacillus casei* ATCC 4646 is even lower, at approximately 3.2 (1, 2). These differences in acid tolerance seem to be important for cariogenicity (11). *S. mutans* and *Streptococcus sobrinus* are considered to be the major cariogenic species in plaque (8). Certainly, their abilities to carry out glycolysis at a pH as low as 4.0 must contribute to the acid challenge to teeth after exposure to sugar. The minimum pH in plaque in vivo is probably due mainly to their activities. *Lactobacillus* organisms make up only a small part of the plaque population. However, because of the stratification of plaque, they may be active at the leading edge of caries lesions (3) and may reduce the pH there to values even lower than 4.0.

We have previously (1) carried out a comparative study of acid tolerance focused on plaque streptococci, primarily *S. mutans* GS-5, a relatively acid-tolerant organism, and *Streptococcus sanguis* NCTC 10904, a relatively intolerant organism. This comparative work has now been extended to an even more tolerant bacterium, *L. casei* ATCC 4646, and an intolerant bacterium, *A. viscosus* OMZ105E.

MATERIALS AND METHODS

Bacteria and culture media. *L. casei* ATCC 4646 was obtained from the American Type Culture Collection, Rockville, Md. It was originally isolated from a carious lesion. *A. viscosus* OMZ105E was obtained from W. H. Bowen of the Department of Dental Research, University of Rochester. Cultures were maintained with weekly transfer on tryptic soy agar and preserved by lyophilization. They were grown for experiments in static cultures at 37°C in TGM medium (14) containing 3% (wt/vol) tryptone from Oxoid Laboratories (Basingstoke, United Kingdom), 1% (wt/vol) glucose, and 1 g of Marmite per liter, a commercial yeast extract from Marmite, Ltd., Montreal, Quebec, Canada.

Glycolysis assays. Cells were grown in TGM medium until the late exponential phase of culture growth. They were harvested by centrifugation in the cold, washed once with

* Corresponding author.

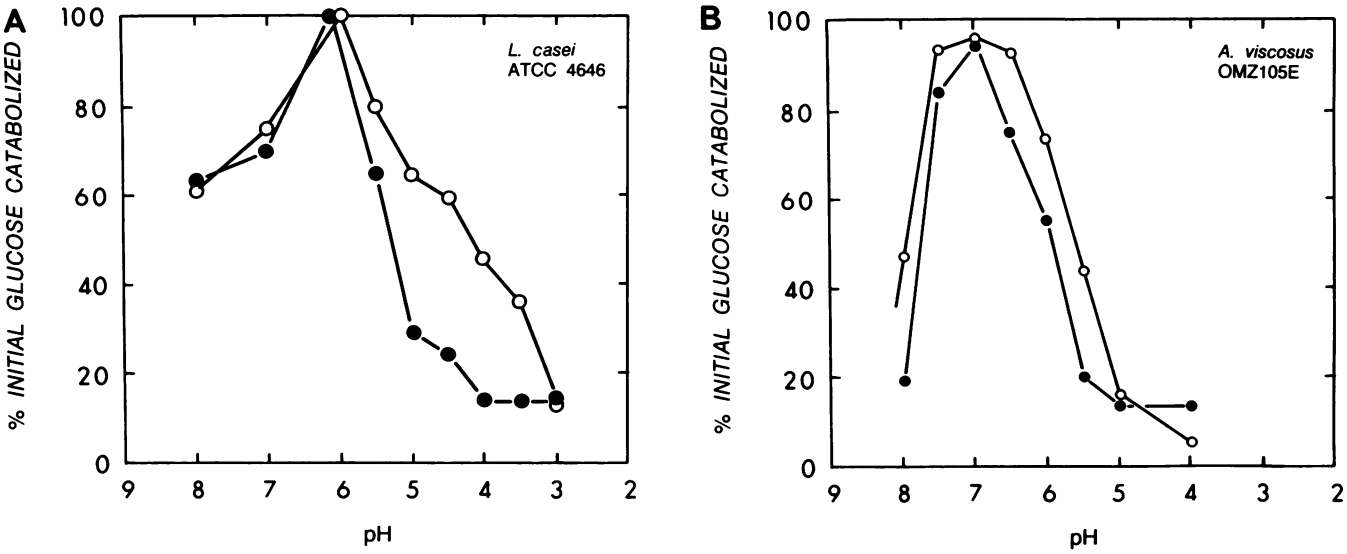

FIG. 1. Activity-pH profiles of normal cells (○) and cells treated with 40 μM gramicidin (●) for *L. casei* ATCC 4646 (A) and *A. viscosus* OMZ105E (B). The data presented indicate the percentage of the glucose added initially that was catabolized after 2 h at 37°C.

deionized water, and incubated for 30 min at 37°C in 20 mM potassium phosphate buffer (pH 7.2) with 1.0 mM $MgCl_2$ to deplete endogenous reserves. They were centrifuged again and suspended in 100 mM potassium phosphate buffer at the desired pH with 1.0 mM $MgCl_2$ and 13.9 mM glucose. Duplicate tubes were prepared for each pH tested, and 40 μM gramicidin was added to one of the tubes. The suspensions were incubated at 37°C, and at intervals, samples were removed and centrifuged rapidly in a microcentrifuge, and supernatant fluids were removed for the glucose assay with glucose oxidase (Worthington Diagnostics, Freehold, N.J.).

Proton permeabilities. The basic procedures described previously (1) were used. Suspensions with 20 mg (dry weight) of cells per ml in 150 mM KCl solution with 1 mM $MgCl_2$ were used. The suspensions were adjusted to the desired pH, and a combination glass electrode connected to a chart recorder was placed in each suspension for continuous monitoring of pH changes. Then the pH of the suspension was reduced by ca. 0.15 pH unit by adding 1 pulse of 10 mM HCl solution with 140 mM KCl from a micropipette. The lowest pH recorded just after addition of the acid was considered to be pH_α. Then, at the end of the experiment, 5% (vol/vol) butanol was added to the suspension to damage the cell membrane and allow for a final rise in pH to pH_ω. The half time for pH equilibration ($t_{0.5}$) was estimated graphically or calculated as the time required for the pH to rise from pH_α to $(pH_\omega - pH_\alpha)/2$.

Mg release. Mg release assays were carried out as described previously (1), with relatively dense suspensions of cells held at a constant pH and an atomic absorption spectrophotometer.

ATPase isolation and assay. Cells were grown in TGM broth with 20 mM DL-threonine until the late exponential phase of growth. They were centrifuged, washed twice in cold, deionized water, and suspended in protoplast buffer containing 75 mM Tris buffer, pH 7.5, with 0.4 mM sucrose and 2 mM $MgCl_2$, supplemented with 1.25 mg of lysozyme per ml. The suspension was incubated at 37°C for 2 h, and the resulting protoplasts were lysed with 0.8 M NaCl. DNase and RNase, 10 μg of each per ml, were added, and the suspension was incubated for 45 min at room temperature with gentle swirling. Membranes were pelleted by centrifugation, washed, and stored as pellets at −20°C. Before use, the pellets were suspended in 50 mM Tris buffer, pH 7.0, with 10 mM $MgSO_4$ and 10% (vol/vol) glycerol.

ATPase activity was assessed in terms of the release of phosphate from ATP and expressed per milligram of membrane protein. The enzyme unit was considered to be that amount required for release of 1 μmol of phosphate per ml per min. The buffer used for ATPase assays was 50 mM Tris maleate with 10 mM $MgCl_2$. The initial ATP concentration was 5 mM. Protein was assayed by the Lowry procedure with bovine serum albumin as the standard (12). Phosphate was assayed by a variant of the Fiske-SubbaRow method with reagents obtained from American Monitor Corp., Indianapolis, Ind.

RESULTS

Gramicidin effects on glycolysis. Gramicidin has proved to be a useful agent for assessing the role of the cell membrane in acid tolerance. It forms channels in membranes through which protons can move; thus, it effectively short-circuits transmembrane proton currents, as shown by the increase in proton permeabilities of treated cells (1). Figure 1 shows the effects of 40 μM gramicidin on glycolysis by intact cells of *A. viscosus* OMZ105E and *L. casei* ATCC 4646 over a range of pHs. Gramicidin had its most striking effects on *L. casei* at a low pH, with a maximum shift to increased acid sensitivity of about 1.5 pH units. The optimal pH for glycolysis by cells of *L. casei* ATCC 4646 was about 6.0, seen here in terms of the percentage of the initial glucose added to the suspensions that was degraded after 2 h of incubation. Gramicidin had little effect at the optimal pH or at a higher pH. However, it markedly changed the acid sensitivity of glycolysis at a lower pH.

The optimal pH for glycolysis of cells of *A. viscosus* was about 7.0. Gramicidin was somewhat inhibitory for glycolysis at all tested pHs. The effect of the antibiotic at a lower pH was somewhat more pronounced, but the maximum change in acid sensitivity was only about 0.5 pH unit.

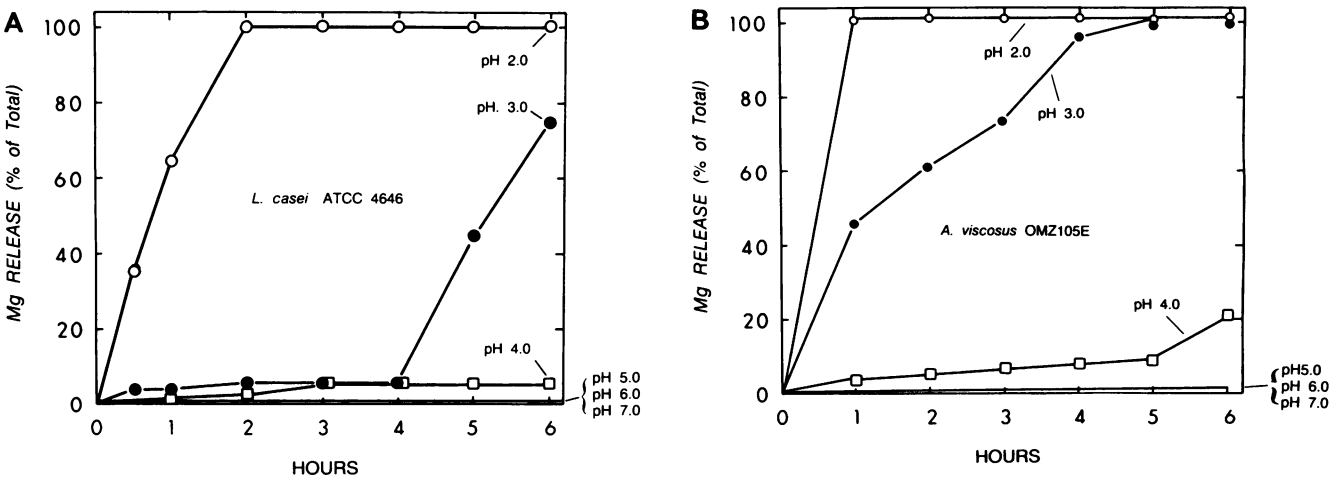

FIG. 2. Acid damage to membranes of *L. casei* ATCC 4646 (A) and *A. viscosus* OMZ105E (B), indicated by magnesium release from cells in suspensions at the indicated constant pH. Total magnesium contents were 66.4 and 75.0 μmol/g (dry weight) of cells for *L. casei* and *A. viscosus*, respectively.

It is readily apparent in the figure that glycolysis by *A. viscosus* is less acid tolerant than that by *L. casei*. The pH at which glycolysis by *A. viscosus* was 50% of the maximal value was 5.7, and this value was shifted to about 5.9 by gramicidin. The pH at which glycolysis by *L. casei* was at about 50% of the maximal was 4.1, and this value was shifted to about 5.3 by gramicidin.

Magnesium release in acid media. Exposure to very acid conditions can result in major damage to cell membranes and release of magnesium from intact cells (1). Generally, the damage is lethal. *L. casei* cells were more resistant to this type of damage than were *A. viscosus* cells (Fig. 2). Suspension of either organism in medium with a controlled pH of 2.0 resulted in release by about 1 h of essentially all of the magnesium detectable by atomic absorption spectrophotometry of ashed cells. When cells of *A. viscosus* were placed in a medium with a controlled pH of 3.0, magnesium release was again complete but occurred somewhat slower than at a pH of 2.0. When *L. casei* cells were placed in the same medium at pH 3.0, there was almost no release of magnesium for 4 h, but then release did occur. Neither organism showed appreciable release of Mg at pH 4.0, 5.0, 6.0, or 7.0 over a 6-h period at room temperature. Although these data indicate a difference between the organisms, this difference is presumably not the main basis for differences in acid tolerance of glycolysis because of the very low pH required for appreciable release of Mg.

Proton permeabilities. Previously, we found (1) that cells of *S. mutans* GS-5 had lower permeabilities to protons at a low pH than did cells of the less acid-tolerant *S. sanguis* NCTC 10904. An even greater difference was found between *A. viscosus* and *L. casei* (Fig. 3). Cells of *L. casei* were least permeable to protons added as HCl at a suspension pH of about 4.0, whereas cells of *A. viscosus* had minimal proton permeability at a pH of about 6.0. Dicyclohexylcarbodiimide (DCCD) markedly increased proton permeabilities of both organisms. DCCD is a specific inhibitor of proton-translocating ATPases of bacteria, and the data indicate a role for the ATPases in moving inflowing protons back out of the cell. A convenient measure of proton permeability is the half time for pH equilibration, that is, the time required for the pH to rise from pH_α, the initial pH after acid addition, to $(pH_\omega - pH_\alpha)/2$, where pH_ω is the pH after addition of butanol to the cells to allow for complete pH equilibration. The $t_{0.5}$ values for the suspensions used to obtain the data of Fig. 3 were 18.1 and 12.0 min for control and DCCD-treated cells of *L. casei*, respectively, and 12.7 and 2.2 min for control and DCCD-treated cells of *A. viscosus*, respectively.

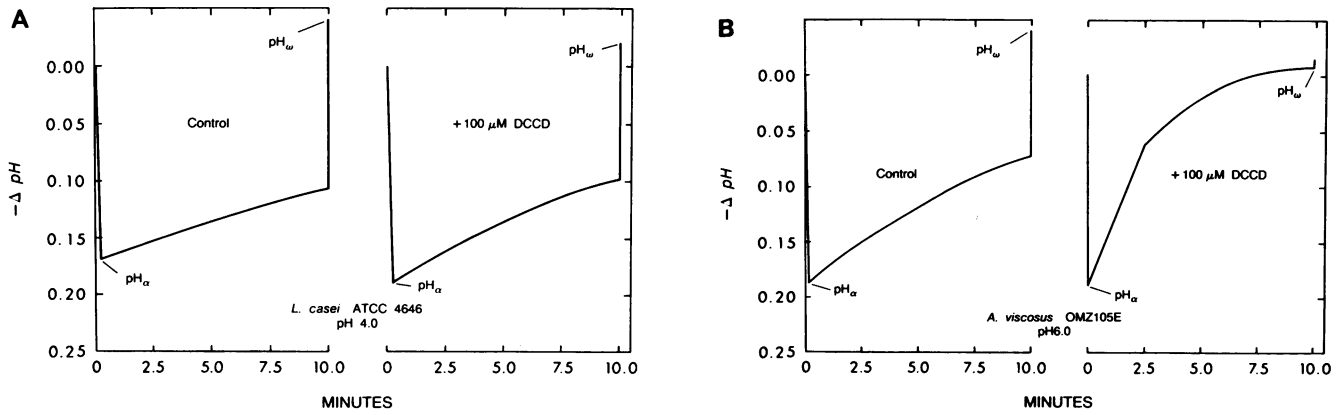

FIG. 3. Proton uptake by cells of *L. casei* ATCC 4646 (A) and *A. viscosus* OMZ105E (B) suspended in solutions containing 150 mM KCl and 1.0 mM $MgCl_2$ after an acid pulse.

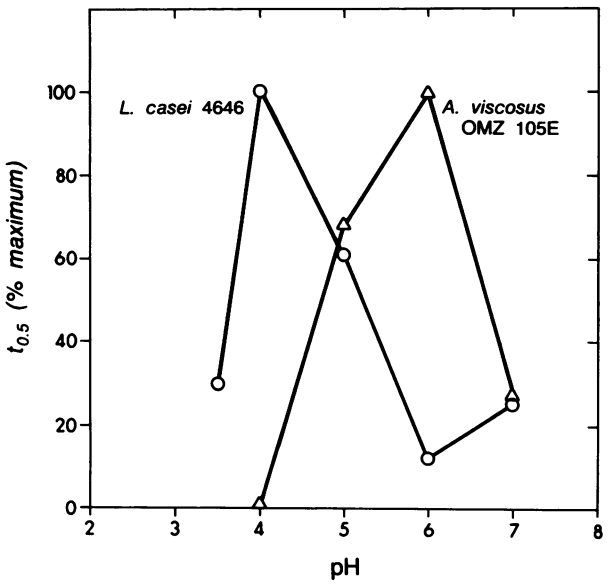

FIG. 4. Proton permeabilities of cells of *L. casei* ATCC 4646 and *A. viscosus* OMZ105E expressed in terms of the $t_{0.5}$ values at the indicated pH. Maximum $t_{0.5}$ values were 24.4 min for *L. casei* and 37.6 min for *A. viscosus*.

Figure 4 presents data on $t_{0.5}$ values for both organisms over a range of pHs determined in a single experiment. At pH 5.0, 6.0, or 7.0, *A. viscosus* cells were actually less permeable to protons than were *L. casei* cells. However, at a pH 4.0, *L. casei* cells had minimal permeability to protons, whereas protons entered *A. viscosus* cells so rapidly that a $t_{0.5}$ value could not be assessed. Even at pH 3.5, *L. casei* cells had a $t_{0.5}$ value of 7.1 min and so would be expected to be able to maintain a ΔpH across the cell membrane.

ATPases. The effects of DCCD on proton permeabilities indicate the involvement of proton-translocating ATPases in transmembrane proton currents. Cell membranes were isolated from both organisms by procedures developed previously (1). Activity-pH profiles for ATPases associated with membranes are presented in Fig. 5. The enzyme of *L. casei* had an optimal pH of approximately 5.0, well below the optimal pH of about 7.0 for the *A. viscosus* enzyme. Moreover, under optimal conditions, *L. casei* membranes were able to catalyze hydrolysis of approximately 3.29 μmol of ATP per min per mg of membrane protein (at pH 5.0 with 5 mM ATP and 10 mM Mg), while membranes of *A. viscosus* were able to hydrolyze only about 0.06 μmol of ATP per min per mg of membrane protein (at pH 7.0 with 5 mM ATP and 10 mM Mg).

Fluoride effects. Both test organisms are considered to be relatively resistant to fluoride. However, because *A. viscosus* is also relatively acid sensitive, it was not possible to assess fluoride sensitivity at the low pH at which fluoride effects are most severe. In pH-drop experiments, control suspensions of *A. viscosus* with excess glucose had a final, minimum pH of about 4.9. However, final pHs for suspensions with 1.0, 2.5, 5.0, 10.0, and 20.0 mM added KF were, respectively, 5.4, 5.6, 6.0, 6.1, and 6.6. Thus, about 5 mM fluoride was required to bring about a sufficient increase in acid sensitivity so that the final pH of a glycolyzing suspension was 6.0. This concentration is essentially the same as that obtained earlier for *S. mutans* GS-5 (5). Thus, although cells of *S. mutans* GS-5 in dense suspensions can lower the pH to about 4.0 normally, addition of 5 mM fluoride sensitizes the bacteria so that the final pH is about 6.0, even with excess glucose present. Overall, it seems that *A. viscosus* is not inherently more resistant to fluoride than is *S. mutans*. It simply operates only at higher pHs because of its acid sensitivity.

L. casei did appear to be inherently more resistant to fluoride than was *S. mutans* GS-5. The final pH in control suspensions of *L. casei* ATCC 4646 was about 3.3. Final pHs for suspensions with 2.5, 5.0, and 10.0 mM added KF were, respectively, 4.4, 4.8, and 5.0. The fluoride concentration required so that the final pH for a glycolyzing suspension of *S. mutans* GS-5 is 5.0 was found (5) to be about 1.1 mM.

We have found that the membrane ATPase of *L. casei* ATCC 4646 is more resistant to the inhibitory action of fluoride than the enzyme of *S. mutans* GS-5 is (S. V. W. Sutton, Ph.D. thesis, University of Rochester, Rochester, N.Y., 1987). Fluoride concentrations for 50% inhibition of the initial rate of hydrolysis of ATP at the optimal pH were about 3 and 25 mM for membranes of *S. mutans* GS-5 and of *L. casei* ATCC 4646, respectively. The comparable value for membranes of *A. viscosus* OMZ105E was found to be about 20 mM. Thus, the enzyme of *A. viscosus* appeared to have a relatively low sensitivity to fluoride compared with that of *S. mutans* GS-5, even though cells of the two bacteria appeared to have about the same sensitivity to fluoride in terms of reduced acid tolerance and inhibition of glycolysis.

Despite the resistance to fluoride shown by glycolyzing cells of *L. casei* or the ATPase of isolated membranes, fluoride still was found to affect proton permeabilities. For example, in an experiment with *L. casei* similar to the one from which the data of Fig. 3 was obtained, the proton permeabilities at pH 7.0, 6.0, 5.0, 4.0, and 3.5 in terms of the $t_{0.5}$ values were 3.8, 7.3, 25.3, 35.0, and 11.4 min, respectively. Addition of 1 mM KF to the suspensions lowered the $t_{0.5}$ values to 1.2, 3.6, 12.9, 28.8, and 8.4 min, respectively. DCCD (100 μM) was more effective than fluoride, and the comparable $t_{0.5}$ values were <1, 5.4, 1.8, 13.0, and 4.6 min, respectively. In essence, fluoride did act to increase proton permeability, even though the ATPase of the organism is relatively resistant to fluoride. Presumably, the action is due mainly to HF acting as a carrier for protons across the cell membrane or as an inhibitor of ATP synthesis.

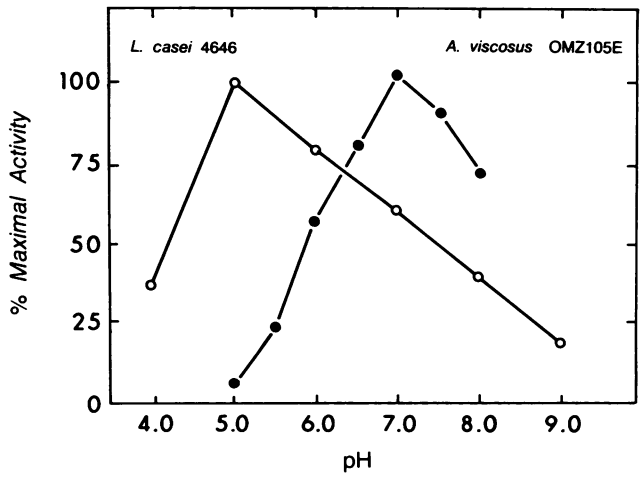

FIG. 5. Activity-pH profiles for ATPases of membranes isolated from cells of *L. casei* ATCC 4646 and *A. viscosus* OMZ105E. The maximal specific activities of the membranes were 3.29 U/mg of membrane protein for *L. casei* and 0.06 U/mg of membrane protein for *A. viscosus*.

DISCUSSION

Over the past few years, a reasonably complete view of the general physiological bases for differences in acid tolerance among common bacteria isolated from supragingival plaque has been developing. There are still many missing details, but the general aspects are becoming clear. The major differences in acid tolerance seem to be related to differences in membrane physiology (1, 2). In view of the effects of the specific inhibitor DCCD, it appears that proton-translocating ATPases play major roles in moving protons out of cells and in decreasing their net permeabilities to protons. Fluoride, the major anticaries agent in use today, acts to short-circuit proton currents across the cell membrane, at least partly because HF acts as a carrier to bring protons into the relatively alkaline cytoplasm of the cell, and possibly also because fluoride inhibits membrane ATPases, in addition to inhibiting enzymes such as enolase (1, 2).

The organisms used for the present study were chosen because they are extreme among plaque bacteria; *L. casei* is the most acid-tolerant organism in plaque, and *A. viscosus* is among the most acid sensitive, at least of the gram-positive organisms. The peculiarities of *L. casei* associated with acid tolerance include somewhat greater resistance of membranes and enzymes to acid damage, higher specific activities of ATPases in the cell membrane, a lower pH optimum of the membrane-associated enzyme, and greater capacities of cells to expel protons at low pH. The contrast between *A. viscosus* and *L. casei* stands out clearly in relation to each characteristic in the data presented in this paper. *S. mutans* is relatively acid tolerant in relation to other plaque bacteria but not as tolerant as *L. casei* was. Membranes of *S. mutans* were found previously (16) to have about 1 U of ATPase activity per mg of protein, compared with about 3 U for *L. casei* and about 0.06 U for *A. viscosus*. The optimal pH of the enzyme from *S. mutans* was about 6.0, and the pH at which intact cells were minimally permeable to protons was about 5.0 (1). In general, it appears that for all of the organisms studied, the pH for minimal proton permeability of intact cells is at least 1 pH unit more than the optimal pH for the membrane ATPase. This difference is not surprising because the ATPase is located on the cytoplasmic face of the cell membrane, and cells are able to maintain a ΔpH across the membrane, with the cytoplasm relatively alkaline when the cells are in acid medium (10). *S. sanguis* NCTC 10904 has been found to be more acid sensitive than *S. mutans* and is similar to *A. viscosus* in having membrane ATPases with a relatively high optimal pH for activity between 6.0 and 7.0. Intact cells of *S. sanguis* NCTC 10904 had minimal permeability to protons at a relatively high pH of about 7.0. Other elements may be involved in acid tolerance, for example, the arginine deiminase system of *S. sanguis* (13). However, the evidence available indicates that proton-translocating ATPases play a major role and that acid tolerance depends on both the amount of the enzyme in the cell membrane and its biochemical characteristics, especially the pH optimum for activity.

The cyclic acidification of dental plaque associated with the intermittent feeding pattern of the host presumably contributes to the diversity of the plaque community in regard to acid tolerance. The various members of the community would have intermittent activities depending on the pH of the environment and their acid tolerance. There have been indications that fluoride may favor *Lactobacillus* organisms in plaque because of their high tolerance to fluoride (3). There is evidence that *S. mutans* can become physiologically adapted to function in the presence of levels of fluoride inhibitory to unadapted bacteria (7). However, *Lactobacillus* organisms also are capable of this same sort of adaptation (8). Overall, it appears that the acid tolerance is of major importance in the ecology of dental plaque and in the effects of fluoride on the plaque community and that tolerance depends on membrane properties, mainly the amounts and characteristics of proton-translocating ATPases.

ACKNOWLEDGMENTS

We thank Karl Stoler for his help in characterizing the ATPases of *A. viscosus*.

This work was supported by Public Health Service grants RO1-DEO6127 and P50-DEO7003 from the National Institute of Dental Research.

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