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Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to Acid Stress in Continuous Culture

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Streptococcus mutans **GS-5 and IB1600 adapted to growth in acidic environments in continuous culture at slow (generation time = 8.3 h) or fast (generation time = 2.4 h) rates of growth in complex medium with a restricted glucose supply. The extent of adaptation was indicated by changes in minimum pH values attained by harvested cells suspended in dense suspensions with excess glucose and by increased levels of ATPase activity assayed in permeabilized cells. Also, adapted cells better withstood potentially lethal acidification. Cells harvested from cultures growing at pH values close to 5 reduced suspension pH to lower values than cells from cultures maintained at pH 7. Cells from pH 6 cultures were intermediate. The IB1600 strain had a higher level of constitutive acid resistance than the GS-5 strain and also was better able to adapt to growth in acidified media. Both had less adaptive capacity than *Enterococcus hirae* ATCC 9790. Adaptation occurred rapidly, mainly within a single generation in continuous culture, while deadaptation occurred more slowly over multiple generations. The capacity of *S. mutans* to adapt to acid conditions is likely to be important in the ecology of dental plaque and also for the cariogenicity of the organism.**

The capacities of microorganisms to adapt to new environments can be important in a variety of situations. For example, yeast cells can adapt to function in the acidified environment of fermenting wort by modulating the activity of the plasma membrane, proton-translocating ATPase (9, 10) and possibly also the vacuole ATPase (17). Microorganisms involved in infections of the animal body also often have to adapt to acid environments. For example, *Salmonella typhimurium* is a facultative intracellular parasite able to survive in the acid environment of the phagolysosome, where the pH may be as low as 3. The adaptation appears to involve multiple genes as indicated by changes in extracted proteins separated by two-dimensional gel electrophoresis (11). Moreover, the F_1F_0 proton-translocating ATPase of the organism appears to play a key role in adaptation as indicated by the inability of *unc* mutants to undergo the adaptive response. A direct correlation between acid tolerance and virulence was proposed by Foster and Hall (11).

Previously, Goodson and Rowbury (13) had shown that the closely related bacterium *Escherichia coli* can become habituated to acid environments when growing at low pH and that habituation results in increased resistance to acid killing in media with a pH of 3.0 or 3.5. *E. coli* appears to be somewhat less adaptable than *S. typhimurium* (15) but does produce a series of acid shock proteins (14). Some years earlier, Gale and Epps (12) had shown that *E. coli* could adapt to both acid and alkaline growth conditions by changing the cell levels of decarboxylase and deamidase enzymes specific for amino acids. Organisms such as *Streptococcus sanguis* also can be protected against acid killing by the arginine deiminase system (8), which catalyzes production of ammonia from arginine.

Tolerance of acid environments is of major importance in the ecology of dental plaque and also in the pathogenesis of dental caries, which involves acid dissolution of tooth mineral. We have shown previously that acid tolerance can be related directly to levels of F_1F_0 ATPase per milligram of protein in membranes of representative bacteria from supragingival dental plaque (3, 4). Tolerance could be related also to pH optima for the membrane-associated enzymes in that the more acid-tolerant organisms have enzymes with lower pH optima. Thus, for example, membranes isolated from the very tolerant plaque bacterium *Lactobacillus casei* ATCC 4646 had approximately 3.3 U of ATPase per mg of protein and a pH optimum for ATP hydrolysis close to 5.0. In contrast, membranes from the acid-intolerant plaque bacterium *Actinomyces viscosus* OMZ 105E had ATPase levels of only about 0.06 U/mg and an optimal pH for activity somewhat above 7.0. Similar differences were obtained in studies with cells permeabilized by freezing and thawing and treatment with toluene rather than with isolated membranes. The cariogenicities of these plaque bacteria appear to depend on acid tolerances, mainly because the acid attack on the tooth which leads to caries is directly related to both the extent and duration of dental plaque acidification. The water solubility of dental enamel increases sharply with decreasing pH below about 5.5 (22), and so acid exposure at a pH as low as 4, or even somewhat lower in cariogenic plaque, can result in enamel dissolution which does not become fully repaired during the alkalinization phase of the plaque acid-base cycle. The abilities of organisms such as *L. casei* and *Streptococcus mutans* to carry out glycolysis at a pH of 4.0 or less appear to be of major importance in the caries process. *S. mutans* is generally considered to be the major cariogenic organism in plaque because it not only is acid tolerant but it also is successful in becoming numerically significant in the total plaque population, especially in individuals with high sugar consumption and prolonged plaque acidification (20).

The new data presented in this report indicate that *S. mutans* not only has constitutive acid tolerance but is also capable of developing adaptive tolerance during prolonged growth at low pH. Clearly, this latter type of adaptation would be expected to affect cariogenicity, especially in plaque in which acidification is prolonged. In this study, we

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compared the adaptation of *S. mutans* developed during growth in continuous culture at low pH with that of *Enterococcus hirae* because the enterococcus is known to be able to respond to cytoplasmic acidification with increased F_1F_0 ATPase activity (1, 18).

MATERIALS AND METHODS

Organisms and growth conditions. *S. mutans* GS-5 and *E. hirae* ATCC 9790 were maintained in our culture collection; *S. mutans* IB1600 was obtained from D. Zero of the Eastman Dental Center, Rochester, N.Y. The APR-11 strain of *E. hirae* ATCC 9790 with defective catabolite repression has been described previously (7, 21). All cultures were grown at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 3 g of glucose per liter. Cultures were propagated in a Bioflow Chemostat (New Brunswick Corp., New Brunswick, N.J.) stirred at 250 rpm, and pH was regulated by the addition of 2 M KOH solution. Glucose depletion from the medium was assayed with glucose oxidase. The specific growth rate (μ) was considered to be equal to the dilution rate (D) of medium into the vessel at steady state. Generation times were then estimated by use of the relationship $T_G = \ln 2/D$, where ln 2 is 0.693 and T_G is the generation time.

Dry weight determination. Samples of 25 ml of cultures after at least 10 generations under the specified conditions were aseptically removed from culture vessels through a vacuum sampling port. Cells were harvested by centrifugation in the cold, washed once with deionized water, and transferred in a small volume of deionized water to a tared aluminum weighing pan. The cells were dried overnight at 100°C and weighed.

Cell permeabilization. Samples of 25 ml of cultures were centrifuged in the cold, and cells from each sample were resuspended in 2.5 ml of 75 mM Tris-HCl buffer (pH 7.0) with 10 mM $MgSO_4$. Toluene (250 μl) was added to each cell suspension prior to vigorous vortex mixing and incubation for 5 min at 37°C. Each cell suspension was then subjected to two cycles of freezing in a dry ice-ethanol bath and thawing at 37°C. Permeabilized cells were harvested by centrifugation. They were then resuspended in 1.0 ml of 75 mM Tris-HCl buffer (pH 7.0) with 10 mM $MgSO_4$. The suspension was quickly frozen in a dry ice-ethanol bath and stored at −70°C.

ATPase assays. A 75-μl sample of permeabilized cell suspension was added to 3.0 ml of 50 mM Tris-maleate buffer (pH 6.0) with 10 mM $MgSO_4$, and the mixture was warmed to 37°C. The ATPase reaction was initiated by the addition of 30 μl of 0.5 M ATP (pH 6.0). Samples of 50 μl were removed and assayed for inorganic phosphate liberated from cleavage of ATP by the Fiske-SubbaRow method as modified by Weisman and Pileggi (23) with reagents from American Monitor Co. (Indianapolis, Ind.). ATPase activities were expressed as micromoles of phosphate released from ATP per gram of cell dry weight. The standard unit of ATPase activity is 1 μmol of phosphate released per min.

Glycolytic pH minimum. A culture sample containing 10 mg (dry weight) of cells was harvested by centrifugation, and the cells were washed with 10 ml of a solution containing 50 mM KCl and 1 mM $MgCl_2$. The cells were then resuspended in dense suspension containing 2 mg (dry weight)/ml in 50 mM KCl solution with 1 mM $MgCl_2$. The suspension was titrated with KOH to a pH just above 7.2. Glucose was added to a final concentration of 55.6 mM, and the fall in pH at room temperature was monitored with a Beckman 45 pH meter (Irvine, Calif.). Timing of the pH fall was initiated when the pH had fallen to 7.2.

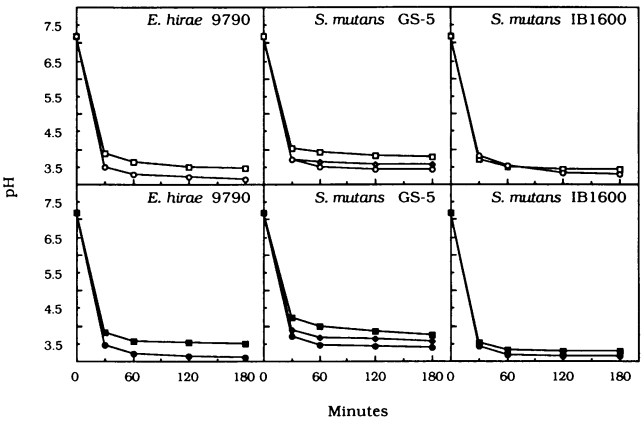

FIG. 1. Glycolytic pH profiles of *E. hirae* 9790, *S. mutans* GS-5, and *S. mutans* IB1600. Cells were grown in continuous culture for approximately 10 generations with generation times of either 8.3 h (□, ◇, ○) or 2.4 h (■, ◆, ●) at pH 7.0 (□, ■), 6.0 (◇, ◆), and 5.0 (○, ●).

Acid killing. Cell suspensions were prepared as described above for determinations of minimal glycolytic pH values, but they were then acidified to pH 2.5 with a 50 mM HCl solution. The acidified suspensions were stirred continuously at room temperature. Samples were removed at 15-min intervals and placed in diluents of 1% (wt/vol) peptone broth (Difco), and 0.1-ml samples of the diluted suspensions were plated on the surfaces of plates containing brain heart infusion agar. The plates were incubated at 37°C for at least 24 h to allow for full growth of colonies before counting for viability assessments.

RESULTS

Adaptive acid tolerance. The most direct indicator that oral streptococci growing for prolonged periods in continuous culture at low pH can adapt is seen in the plots presented in Fig. 1 and the summary data of Table 1. Cells harvested from acidified cultures and resuspended in dense suspension with excess glucose were able to drop suspension pH to lower values than were cells harvested from cultures maintained at pH 7. The plots shown in Fig. 1 are standard acid-drop plots used to estimate minimum pH values at which cells can carry out glycolysis. The cells used here were not so-called runoff cells collected from the culture effluent but were from harvests directly from the main culture vessel. The cells were suspended at a concentration of 2 mg (dry weight)/ml in a solution of 50 mM KCl and 1 mM $MgCl_2$ so that their acid tolerance would be near maximal. Changes in suspension pH after the addition of 55.6 mM glucose are shown. Glycolysis stopped because of acidification, not because of substrate depletion, and even after 180 min, neutralization of the produced acid resulted in a new round of glycolysis. The pH minima obtained in this way are approximately equal to those obtained by the more tedious method of suspending cells in buffers at various pHs and determining the minimum pH at which glycolysis occurs. In Fig. 1, then, lower final pH values indicate higher acid tolerance of glycolysis.

The data for *S. mutans* GS-5 (Fig. 1 and Table 1) indicate that cells grown at a slow rate (generation time = 8.3 h) in medium with a controlled pH of 5.3 were able to perform

TABLE 1. Acid-adaptive response of streptococci

Organism	Growth pH	Generation time of 8.3 h		Generation time of 2.4 h	
		3-h glycolytic pH[a]	ATPase (μmol of P/g/min)[a]	3-h glycolytic pH	ATPase (μmol of P/g/min)
S. mutans GS-5	7	3.75 (0.24)	32 (3)	3.74 (0.10)	46 (1)
	6	3.56 (0.09)	34 (12)	3.55 (0.12)	68 (5)
	5[b]	3.45 (0.13)	39 (17)	3.38 (0.05)	77 (9)
S. mutans IB1600	7	3.43 (0.02)	34 (8)	3.26 (0.03)	44 (13)
	5	3.27 (0.15)	75 (19)	3.14 (0.01)	104 (34)
E. hirae 9790	7	3.47 (0.08)	79 (18)	3.49 (0.02)	57 (22)
	5	3.14 (0.01)	180 (95)	3.10 (0.10)	161 (96)
E. hirae APR-11	7	ND[c]	ND	3.63 (0.10)	64 (3)
	5[d]	ND	ND	3.74 (0.03)	56 (22)

[a] Mean (standard deviation); $n = 3$.
[b] With a generation time of 2.4 h, the vessel pH stabilized at pH 5.3 ± 0.1.
[c] ND, Not determined.
[d] With a generation time of 2.4 h, the vessel pH stabilized at 5.12 ± 0.02.

glycolysis until the suspension pH had dropped to 3.45. By comparison, cells from cultures maintained at a constant pH of 7.0 were able to reduce the suspension pH only to 3.75, and cells from cultures maintained at a constant pH of 6.0 were intermediate in terms of glycolytic acid tolerance. Cells grown at a faster growth rate (generation time = 2.4 h) showed somewhat greater adaptive ability. Also shown in Table 1 and Fig. 2 are data for F_1F_0 ATPase activities of permeabilized cells prepared from the same cultures used to obtain cells for determination of minimum pHs for glycolysis. The adaptation resulting in lower minimal glycolytic pHs was reflected in increased ATPase activities. However, no major changes in pH optima for activity or pH profiles for activity were detected for ATPases of adapted cells, although the pH profile was somewhat broader in the alkaline range for unadapted cells. The standard deviations indicated in Table 1 reflect mainly the experiment-to-experiment variations in the final pH and do not show that adaptation was variable.

S. mutans IB1600 had greater constitutive acid tolerance than did the GS-5 strain. Thus, even unadapted cells grown at the slower growth rate could lower the pH to 3.43 in glycolyzing suspensions. However, adapted cells could reduce the pH to an even lower value of 3.27. The adaptation was evident also for cells grown at the faster rate, and fully adapted cells were able to reduce the pH in glycolyzing suspensions to a low value of 3.14. The changes in ATPase values associated with adaptation were more pronounced for cells grown at the faster rate than for those grown at the slower rate.

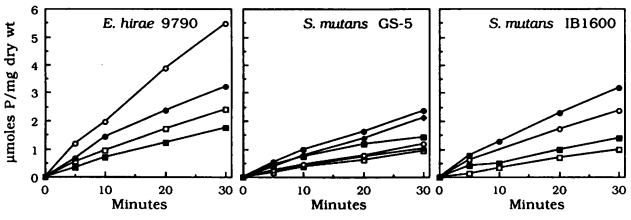

FIG. 2. ATPase activities of permeabilized cells. Cells were grown in continuous culture for approximately 10 generations with generation times of either 8.3 h (□, ◇, ○) or 2.4 h (■, ◆, ●) at pH 7.0 (□, ■), 6.0 (◇, ◆), and 5.0 (○, ●).

Adaptation of *E. hirae* ATCC 9790 was even more pronounced, especially in terms of increased ATPase activity. The constitutive acid tolerance of the organism grown at either the faster or the slower rate was intermediate in relation to the two strains of *S. mutans*. However, the enterococcus had greater capacity to adapt to acid conditions than did the plaque streptococci.

The APR-11 strain of *E. hirae* is a variant of the ATCC 9790 strain with defective catabolite repression and reduced activity of the glucose-specific enzyme II of the phosphotransferase system. It had lower constitutive acid tolerance than the parent organism and, moreover, did not develop increased tolerance during prolonged cultivation in media with a pH of 5.12.

Adaptive resistance to acid killing. The acid adaptation, indicated by reduction in pH minima for glycolysis and increased ATPase activities, was reflected also in enhanced resistance to acid killing. An example is presented in Fig. 3 for *S. mutans* GS-5. Cells from cultures grown for prolonged periods at a pH of 5.3 were better able to withstand lethal acidification to a pH of 2.5 than were cells from continuous cultures maintained at a pH of 7.0.

Kinetics of adaptation. The time course of adaptation and deadaptation of *S. mutans* GS-5 to acidification is shown by the plots presented in Fig. 4. A culture of the organism was allowed to stabilize for 10 generations at a controlled pH of 7.0 with a generation time of 2.4 h. A sample of the culture was taken for determination of zero time minimum glycolytic pH and ATPase activity. The pH controller of the chemostat was switched from 7.0 to 5.0, and the cells in the culture were allowed to lower the pH by acid production. Adaptation was evident after only about one generation as indicated by the decline in minimum glycolytic pH from 3.63 to 3.43. Further adaptation occurred until at the sixth generation after acidification, the minimum pH for glycolysis had fallen to 3.30. Also, the ATPase activity had increased from 36.5 to 105.2 μmol of phosphate per min per g (dry weight).

After the sixth generation, the pH controller was switched back to 7.0. There was then a slow, steady increase in the minimum pH for glycolysis and also a decrease in ATPase

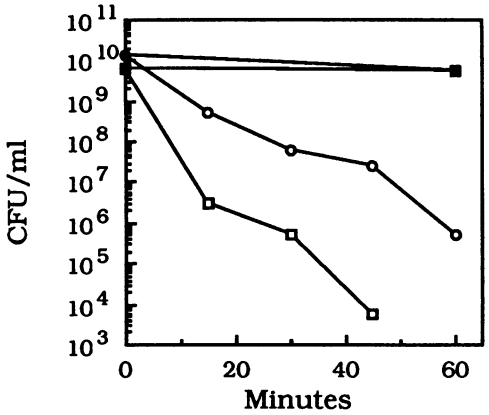

FIG. 3. Survival of acid-adapted and unadapted cells of *S. mutans* GS-5 during acid challenge. A thick suspension of cells was prepared from a harvest from a steady-state chemostat held at pH 7.0 (□, ■) or pH 5.0 (○, ●) and either titrated to pH 2.5 (□, ○) or not titrated to act as a control (■, ●).

activity. However, complete deadaptation occurred over approximately 15 generations.

When cells were suspended in buffers of pH 7.0 or 5.0 and incubated at 37°C for 3 h, no adaptation could be detected. In other words, it appeared that growth was necessary for adaptation.

DISCUSSION

The data presented in this report indicate clearly that *S. mutans* and *E. hirae* can adapt physiologically to prolonged growth in continuous culture at pH values close to the minimum for growth. The adaptation appears to be progressive, becoming more marked at lower and lower pH values of the growth medium, and reversible, at least over a number of generations. In all, the adaptation did not appear to be due to selection of variants in the population with greater constitutive acid resistance. Most of the adaptation occurred rapidly, in about one generation, as indicated by the data presented in Fig. 4. This speed of adaptation argues against genetic selection. Moreover, it may be of advantage to the bacteria to be able to adapt rapidly to acidification, not only

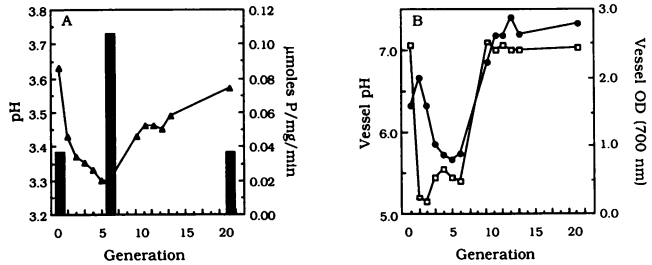

FIG. 4. Acid adaptation kinetics of *S. mutans* GS-5 in continuous culture. Cells were allowed to come to steady state (10 generations) at pH 7.0, with a generation time of 2.4 h. The vessel was then sampled, and that time point is designated generation zero (0). The pH controller was then set to a pH of 5.0. After the sixth-generation sampling of the vessel, the pH was reset to 7.0. The 3-h minimum pH of glycolysis (△) and ATPase activity (bar graph, ▩) are shown from a representative single experiment in panel A. Vessel pH (□) and culture optical density (OD) at 700 nm (●) are shown in panel B.

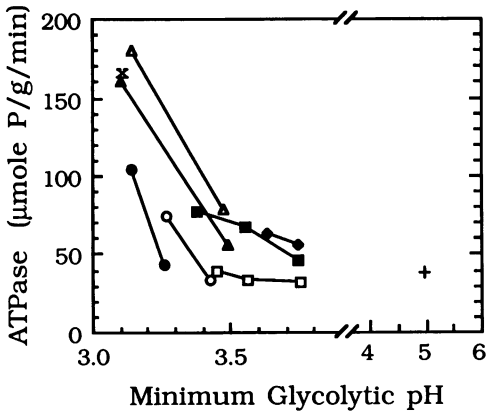

FIG. 5. Relationship between ATPase levels and acid tolerance of glycolysis for lactic acid bacteria. Symbols: □, ■, *S. mutans* GS-5; ○, ●, *S. mutans* IB1600; △, ▲, *E. hirae* ATCC 9790; ◆, *E. hirae* APR-11; +, *S. sanguis* NCTC 10904; ×, *L. casei* ATCC 4646. Open symbols indicate growth with an 8.3-h generation time, and closed symbols indicate growth with a 2.4-h generation time. The points for *S. sanguis* and *L. casei* were derived from data presented in references 3 and 4 for batch cultures.

for improved function in acid media but also to avoid the potentially lethal effects of acidification. Bowden and Hamilton (6) have found that *S. mutans* can survive progressive acidification but is inactivated by rapid acidification, presumably because rapid acidification does not allow for adaptation. The slower deadaptation occurring over a number of generations also appears to reflect not genetic selection but a progressive physiologic process, for example, changing membrane lipid composition, which is known to affect activities of membrane ATPases.

One of the most clearly defined examples of acid adaptation is that of yeast cells. Increased ATPase activity associated with increased acid tolerance of *Saccharomyces cerevisiae* is not due to increased transcription or translation as indicated by determination of levels of mRNA or serological detection of ATPase enzyme (9). Instead, the enzyme is somehow activated, possibly through effects of inhibitory epsilon subunits (16), proteolytic modification (2), or changes in membrane lipid composition. In contrast, Kobayashi and co-workers (18, 19) detected increased ATPase enzyme after adaptation of *E. hirae* by staining with Coomassie brilliant blue G-200 the subunits separated on gels and detecting increased binding of ^{125}I-labeled protein A to complexes of antibody and separated subunits in a Western immunoblot. Presumably, then, for *E. hirae*, adaptation involves increased transcription of genes for F_1F_0 ATPase and increased synthesis of the enzyme.

Current knowledge of the acid-base physiology of oral streptococci suggests that the F_1F_0 proton-translocating ATPase is the major player in maintenance of ΔpH across the cell membrane, with the cytoplasm alkaline relative to the acid environment. The relationship between ATPase levels and acid tolerance in a range of plaque bacteria has been explored recently (3, 4). Figure 5 presents a summary of data obtained in the present study and some data obtained previously. The data from previous studies in which ATPase levels of isolated membranes were assessed were converted to values comparable to those for permeabilized cells by assuming that membranes are 50% protein and that the cell membrane makes up approximately 10% of the dry weight of

the cell. *S. sanguis* NCTC10904 was chosen as an acid-sensitive lactic acid bacterium, while *L. casei* ATCC 4646 was chosen as an acid-tolerant lactic acid bacterium. There clearly are peculiarities of the individual organisms, and there is clearly a growth rate effect. However, it seems that overall there is a hyperbolic relationship between ATPase levels and acid tolerance of glycolysis. Above minimum glycolytic pHs of about 3.5, relatively small changes in ATPase levels are associated with significant changes in acid tolerance of glycolysis. However, rather large changes in enzyme levels are required to lower the minimum glycolytic pH much below 3.5. This sort of relationship seems reasonable since the pH scale is exponentially related to proton concentrations. The adaptability of *E. hirae* is remarkable. The constitutive tolerance of the organism is similar to that of the GS-5 strain of *S. mutans*, but adapted cells have acid tolerance similar to that of *L. casei*.

For *S. mutans*, acid adaptation would be expected to be important for ecological competitiveness because the organism requires an acidified environment to be able to compete effectively with less acid-tolerant organisms such as *S. sanguis* (5). The speed with which the bacterium adapts suggests that at least partial adaptation occurs within the duration of a single exposure of plaque to fermentable carbohydrate and is likely during prolonged or repeated acidification. Very rapid drops in plaque pH may actually preclude adaptation. However, more gradual drops or pH cycles may allow for adaptation, especially since deadaptation was found to occur more gradually than adaptation. Prolonged acidification of plaque may enhance damage to teeth not only by promoting more severe demineralization but also by serving to induce adaptive changes in plaque bacteria allowing them to lower pH values beyond those achievable with unadapted organisms. Thus, acid adaptation may play an important role in the cariogenicity of *S. mutans*.

ACKNOWLEDGMENTS

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