Acid Tolerance of Biofilm Cells of *Streptococcus mutans*†

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*Streptococcus mutans*, a member of the dental plaque community, has been shown to be involved in the carious process. Cells of *S. mutans* induce an acid tolerance response (ATR) when exposed to sublethal pH values that enhances their survival at a lower pH. Mature biofilm cells are more resistant to acid stress than planktonic cells. We were interested in studying the acid tolerance and ATR-inducing ability of newly adhered biofilm cells of *S. mutans*. All experiments were carried out using flow-cell systems, with acid tolerance tested by exposing 3-h biofilm cells to pH 3.0 for 2 h and counting the number of survivors by plating on blood agar. Acid adaptability experiments were conducted by exposing biofilm cells to pH 5.5 for 3 h and then lowering the pH to 3.5 for 30 min. The viability of the cells was assessed by staining the cells with LIVE/DEAD BacLight viability stain. Three-hour biofilm cells of three different strains of *S. mutans* were between 820- and 70,000-fold more acid tolerant than corresponding planktonic cells. These strains also induced an ATR that enhanced the viability at pH 3.5. The presence of fluoride (0.5 M) inhibited the induction of an ATR, with 77% fewer viable cells at pH 3.5 as a consequence. Our data suggest that adhesion to a surface is an important step in the development of acid tolerance in biofilm cells and that different strains of *S. mutans* possess different degrees of acid tolerance and ability to induce an ATR.

The oral microbial flora is under normal circumstances beneficial for the host and prevents colonization by pathogenic species (21). Carbohydrate metabolism by oral streptococci via the glycolytic pathway is of considerable importance to the ecology of the dental plaque biofilm, since the resulting acid formation lowers the plaque pH (6). The degree and rate of the pH fall increase with the concentration of carbohydrate in the food, particularly that of refined sugars, such that the plaque pH can reach values of less than 5.0 and remain at low levels for some time (27). Under these conditions, the low pH can lead to demineralization of the enamel, as well as the selection of strains that are able to adapt and survive in these acidic environments. In this way, an acid-tolerant microflora will emerge which in turn will promote further demineralization and development of caries (5, 21). Considerable research has focused on *Streptococcus mutans* and its involvement in the caries process (5, 19). This notoriety has been due in large measure to its ability to degrade carbohydrates rapidly with the formation of large amounts of acid and its ability to adapt to acid tolerance at low pHs (14, 18, 30). Under acidic conditions, *S. mutans* alters its physiology in a variety of ways in order to survive, including the synthesis of stress-responsive proteins, exhibiting increased glycolytic activity and increased activity of the proton-translocating ATPase regulating intracellular pH (3, 13, 14, 31). These changes are now known to be associated with changes in cellular protein synthesis (15, 31).

Recent research on the initial phase of biofilm formation by *S. mutans* has demonstrated significant changes in protein expression within 2 h after surface contact (34). Thus, we were interested in studying whether this initial 2-h contact resulted in increased acid tolerance comparable to that seen with mature biofilms and whether newly adhered biofilm cells were able to induce an ATR. Furthermore, since strains of *S. mutans* have been shown to be genetically diverse, certain genotypes might show more virulent traits than other genotypes (23). Thus, the further aim of this study was to expand the investigation of the acid adaptability and acid tolerance in newly adhered biofilm cells to different strains of *S. mutans* and to ascertain what role fluoride has on these early events.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *S. mutans* H7 was isolated from dental plaque above a carious enamel surface following plating directly onto a nonselective...
solid agar medium buffered at pH 5.0 (30). S. mutans XAB5 was also isolated from dental plaque and plated onto nonselective solid agar, pH 4.5, while S. mutans UA159 was a kind gift from Dennis Cvitkovitch, University of Buffalo (Toronto, Canada). The S. mutans classification was confirmed by biochemical tests carried out according to Beighton and colleagues (2, 35). The strains produced acid from inulin and had alpha-glucosidase and beta-glucosidase activity, but were negative for N-acetylglucosaminidase and arabinosidase. The growth of each strain in this study was initiated by the transfer of a single colony from blood agar to MM4 minimal medium containing 40 mM phosphate/citrate (P/C) buffer (pH 7.5) and 20 mM glucose (15). For the acid killing and acid adaptation experiments, MM4 was buffered with 40 mM P/C buffer at pH 3.0, 3.5, 4.0, 5.0, 5.5, or 6.0, while the wash medium was MM4 devoid of glucose and P/C buffer. All media were incubated at 37°C with nitrogen plus 5% CO2 prior to each experiment.

Flow-cell biofilm system. The flow cells consisted of two parallel glass slides separated by two 1.6-mm rubber spacers and mounted in a holder which was sealed with O rings and covered by a Perspex lid. The construction followed the original designed at the University of Buffalo (Buffalo, NY) from a modified version of the flow cell described by De Palma (8). Prior to each experiment, the glass slides were boiled in H2O, H2O2, and NH3 (5:1:1) for 15 min, thereafter rinsed in distilled water, and then boiled in H2O, H2O2, and HCl (5:1:1) for another 15 min before a final rinse in water and then twice in 99.9% ethanol. After drying, the slides were sterilized by dry heat, while the rest of the flow cell was sterilized by autoclaving.

Each flow cell had a total volume of 2.1 cm3 and a total surface area of 26 cm2 for biofilm growth. The flow within the flow cell was laminar and was controlled by a peristaltic pump. Valves at the inlet and outlet made it possible to close the flow cell at any time between the times of flow coming in and out from two separate silicone tubes. The flow during the adhesion phase was 42 ml/h (shear flow = 0.06 cm/s) but 200 ml/h (shear flow = 0.27 cm/s) during rinsing, acid stress, and acid adaptation. This flow keeps the pH in the flow cell at pH 7.5 during the rinsing process and lowers the pH in the cell fluid within 5 min at acid killing or acid adaptation. The glucose concentration was also maintained at a constant level by the medium flow to the biofilm chamber. After each experiment, the flow cells were emptied or have flow coming in and out from the two separate silicone tubes.

Fluoride inhibition studies. In order to examine the role of energy metabolism in adaptation to acid tolerance, NaF was added to planktonic and biofilm cells to inhibit glycolysis (13). Initial experiments were undertaken with planktonic cells to determine the NaF concentration required to inhibit metabolism. Washed, log-phase cells were suspended in 20 ml MM4 medium devoid of glucose and incubated for 20 min to deplete any endogenous carbohydrate. Glucose (100 mM final concentration) was then added and the pH of the suspension recorded for 10 min. At this point, 50 μl of 1 M NaF was then added at intervals of 4 min with the pH recorded until there was no further change in pH. The same experiment was conducted with 17.5 mM NaF added before glucose addition to confirm the inhibition of carbohydrate metabolism. Based on the results with the planktonic cells, experiments were carried out with biofilm cells at NaF concentrations between 1 and 750 mM in order to determine a concentration that would preserve cell viability at pH 5.5 but still prevent the cells from adapting to acid tolerance.

Statistical analysis. The results were statistically analyzed using the Mann-Whitney test.

RESULTS

Acid tolerance of 3-h biofilm cells. We have previously shown that 3-day biofilm cells of S. mutans were six orders of magnitude more resistant to acid stress (pH 3.0 for 2 h) than the corresponding planktonic cells (33). In this study, we were interested in determining whether the mere act of a cell contacting the surface to form a biofilm was sufficient in itself to trigger the induction of acid tolerance comparable to that seen with the 3-day biofilms. For this, we prepared individual 3-h biofilms of three different strains of S. mutans and measured their survival after incubation in minimal medium at pH 3.0 for 2 h, using planktonic cells as comparative controls. As seen in Table 1, the 3-h biofilms of all three strains were significantly (P < 0.05) more acid tolerant than the corresponding planktonic cells. The biofilms of S. mutans UA159 (8.2%) were slightly more acid resistant than those of S. mutans H7 (5.1%), while those of S. mutans XAB5 (3.5%) were less resistant than strain UA159; however, there was no statistical difference between the three strains. The acid tolerance of the planktonic cells varied considerably among the three strains, with S. mutans
UA159 (0.01%) being 25- to 200-fold more resistant than strains H7 and XAB5, respectively. This indicates that strain UA159 was inherently more acid tolerant than the other strains; however, the magnitude of the differences between the planktonic and biofilm cells suggests that the surface triggered a more profound adaptive response by *S. mutans* XAB5 (70,000-fold) and *S. mutans* H7 (12,500-fold) than by *S. mutans* UA159 (820-fold). To make sure that the increased acid tolerance in the biofilm cells after adhesion wasn’t due to generation of an acid-tolerant population in the recirculating bacterial suspension, these cells were tested for acid tolerance, and they showed no enhanced acid resistance, with survival figures in the same range as survival of the planktonic cells, with 0.00001% surviving at pH 3.0 for 2 h. The results shown in Table 1 raised the question of whether the increased acid tolerance observed following cellular adherence to the surface would persist if the cells were removed from the surface. This was tested with *S. mutans* H7 by testing the acid sensitivity of biofilm cells removed from the surface and dispersed in MM4 medium at pH 3.0 for 2 h. The presence of a surface was shown to be essential for the enhanced acid tolerance at pH 3.0, since the dispersed cells showed the same survival rate as of planktonic cells, i.e., 0.0009%.

**Survival rates at different pH values.** Since biofilm cells of *S. mutans* were more acid tolerant than planktonic cells at pH 3.0, we were interested in determining whether this difference in tolerance was maintained at higher pH values. Accordingly, survival rates of log-phase planktonic cells and 3-h biofilm cells of *S. mutans* H7 were tested by exposure of the cells to pH values ranging between 6.0 and 3.0, followed by plating of the cells on blood agar. Control cells were maintained at pH 7.5. As seen in Fig. 1, biofilm cells were more acid tolerant than planktonic cells at pH values below 5.0, with no difference in acid tolerance at intermediate pH levels. At pH 5.0, 53.2% of the planktonic cells and 47.4% of the biofilm cells survived, while at pH 4.0, biofilm cells, at 36.5%, were significantly more resistant (*P* < 0.01) than the planktonic cells (11.2%).

![FIG. 1. Comparison of the percent survivors of planktonic and biofilm cultures of *S. mutans* H7 following exposure at different pH values for 2 h and control cells kept at pH 7.5. Survivors were plated on blood agar, and the values represent the means of three independent experiments. Symbols: ▲, biofilm cells; ■, planktonic cells.](http://aem.asm.org/)

![FIG. 2. Effect of a 3-h preincubation in pH 5.5 MM4 medium on the subsequent survival at pH 3.5 for 30 min of biofilm cells of three strains of *S. mutans*. Control biofilms were maintained at pH 7.5. Values represent the percent viable cells as shown by LIVE/DEAD BacLight staining. Symbols: ■, UA159; ▲, H7; □, XAB5.](http://aem.asm.org/)
Nature of the ATR in biofilms. Various studies have demonstrated that a reduction in the pH of planktonic cultures from 7.5 to 5.5 results in the induction of an ATR over a 2- to 3-h period that results in increased survival at lower pH values (22, 30). To determine whether newly adhered biofilm cells induce a similar pH-dependent ATR, 3-h biofilms of the test strains were exposed to MM4 medium buffered at pH 5.5 for 3 h prior to exposure to pH 3.5 medium for 30 min. The biofilm cells were then stained with LIVE/DEAD BacLight viability stain to measure the viability of the cells. As seen in Fig. 2, only 1% of the pH 7.5 control biofilm cells of S. mutans UA159 were viable after exposure at pH 3.5, while 66% of those incubated at pH 5.5 were viable, indicating that adaptation took place in the cells exposed to pH 5.5 medium. The control (pH 7.5) biofilm cells of S. mutans H7 and XAB5 gave similar results at pH 3.5 medium for 3 h; however, survival was lower, with 51% for strain H7 and only 18% for strain XAB5. Analysis of the BacLight “live/dead” staining profiles (Fig. 3) for the various cultures in Fig. 2 indicated that the adaptation period at pH 5.5 did not adversely affect the viability of the cells compared to the controls at pH 7.5. There was no statistical difference in acid adaptability between S. mutans H7 and S. mutans UA159, while there was a difference in acid adaptability between S. mutans XAB5 and the two other strains, with S. mutans XAB5 being less able to adapt at pH 5.5 (P < 0.01).

Role of fluoride in acid adaptation. Previous studies with S. mutans have indicated that energy generation via glycolysis is an important element in survival of acid stress by cells in established 3- to 5-day biofilms (22, 33). To examine this with new 3-h biofilms, we tested the influence of the glycolytic inhibitor, NaF, (13) on acid adaptation and survival of S. mutans UA159 at pH 3.5. NaF (0.5 M) was added to the biofilms during the 3-h adaptation phase with pH 5.5 medium, followed by exposure at pH 3.5 for 30 min. As seen in Fig. 4, biofilm cells incubated in pH 5.5 medium for 3 h were unaffected by 0.5 M NaF, with slightly higher viability (94%) than the control cells (86%) following BacLight staining. There was a distinct difference, however, at pH 3.5 between the cells that had been exposed to NaF and the control cells incubated without fluoride, with the latter exhibiting 77% viability while only 26% of the cells exposed to NaF were viable (P < 0.01). Viable counts obtained by plating the cells removed from the biofilms on blood agar confirmed a large difference in survivors between the nonexposed cells (87%) and those incubated with NaF (12%).

DISCUSSION

A variety of earlier studies have demonstrated the ability of S. mutans to induce an ATR that enhances survival at a low pH, employing both planktonic cultures and mature biofilms (14, 30, 33). Enhanced acid tolerance of the latter cells is in keeping with earlier work on mature biofilms that has demonstrated that biofilm cells are more resistant to environmental stress and antimicrobial agents than their planktonic counterparts (7, 10). On approaching a surface, bacteria encounter environmental conditions different from those in the bulk liquid phase, a situation now known to induce changes in gene and protein expression (12, 32). For example, in 2-h biofilm cells of S. mutans, 33 of the 124 proteins analyzed showed altered expression compared to those of the planktonic cells, with 1 protein expressed only on biofilm cells (34). In the current study, we have shown that adhesion to a surface is also accompanied by increased acid tolerance with enhanced survival up to 70,000-fold greater than that of the corresponding...
planktonic cells. These data suggest that adherence to a surface is an important step in the development of acid tolerance in biofilm cells. In *S. mutans*, proteins of the glycolytic pathway showed enhanced expression in newly formed biofilm cells, which diminished in older biofilms (32, 34). Such changes in protein expression could explain the difference in acid tolerance between mature (41.5%) and newly formed (5.1%) biofilm cells of *S. mutans* (33) (Table 1). These changes in the cellular protein profile could represent the ATR triggered by cycles of acid shock during the development of the mature biofilm. The increased resistance to acid in biofilm cells seems to have two inducing mechanisms, one triggered by surface contact and one by the exposure to an acidic environment.

The surface-induced changes in biofilm cells of *S. mutans* that led to increased acid tolerance without prior adaptation nevertheless disappeared when the cell no longer sensed the presence of the surface. Results with *S. mutans* BM71 growing in a biofilm chemostat system showed that as the biofilms aged over a 7-day period, the acid tolerance increased, and this also was seen with the 5- and 7-day dispersed cells, although the latter cells were still more than 300-fold less acid tolerant than the corresponding biofilms (22). Li and colleagues (18), on the other hand, showed that the dispersal of the biofilms generated by glucose pulsing had little effect on acid tolerance, with dispersed biofilm cells being as tolerant as the intact biofilms. Clearly, these studies demonstrate that acid tolerance increases with high-density biofilms and the cells dispersed from such biofilms also possess greater acid tolerance. Thus, the low density of the 3-h biofilms employed in this study is undoubtedly a factor in the loss of acid tolerance following dispersal of the cells from the biofilms. Furthermore, it is clear from the results of this study that contact with the surface was responsible for the observed acid tolerance, not an acid shock-dependent induction of the ATR. One can also conclude that as the biofilm ages, this tolerance is supplemented with the slow induction of an ATR due to acid shocks in the biofilm that results in the resistance seen with the mature biofilms.

Early studies with planktonic cells of *S. mutans* have demonstrated that the induction of the ATR is accompanied by increased activity for the proton-pumping ATPase and decreased proton permeability of the cell membrane (3, 14). Since the ATR and increased acid tolerance have been demonstrated in this study with newly formed biofilms following incubation at pH 5.5 for 3 h (Fig. 2), one can speculate that similar events are occurring with the cells on the surface. The added feature in this study with the new biofilms is the ability to assess the state of the cellular membranes following treatment with the LIVE/DEAD BacLight stains. The BacLight stain consists of two different nucleic acid stains, SYTO 9 and propidium iodine, with SYTO 9 capable of penetrating all cells and staining them green. Propidium iodine, on the other hand, penetrates only cells with a damaged membrane and stains the cells red. The higher number of viable green cells in the pH 5.5-adapted preparations of all three strains (Fig. 3) than in the pH 7.5 controls indicates that adaptation has resulted in less membrane damage. *S. mutans* has been shown to undergo changes in membrane composition after exposure to pH 5.0, with the membrane profile changing from short-chain fatty acids to long-chain fatty acids. Cells treated with cerulenin to inhibit fatty acid biosynthesis were not able to undergo these changes in membrane composition and were more sensitive to low pH (11, 24). In addition, *Streptococcus sobrinus* 6715, which is known to have minimal acid-adaptive capacity (15), showed only minimal changes in membrane fatty acids (24).

Fluoride, a common additive in toothpaste, inhibits the glycolytic enzyme enolase, which leads to a diminished production of ATP and the acid end products of metabolism (4, 16, 28). Fluoride also inhibits the proton-translocating ATPases, which, coupled with a reduction in the intracellular concentration of ATP, affects the cell’s ability to maintain the intracellular pH (20, 29). Although there has been extensive research on the interaction of fluoride with oral bacteria, little is known about its effect on the acquisition of acid tolerance in newly adhered biofilm cells. In this study, we showed that fluoride inhibited the ability of the cells to induce an ATR (Fig. 4), which in turn made them more acid sensitive. BacLight staining showed that the NaF-treated biofilm cells possessed damaged cell membranes, resulting in a significantly lower (threefold) number of green viable cells than for the control cells, an observation confirmed by sevenfold-lower counts on blood agar. The damage to the membranes clearly made it more difficult for the cells to maintain the cytoplasmic pH at levels permitting essential metabolism. The concentration of fluoride (0.5 M) used in this study was selected because although metabolism was inhibited, the cells nevertheless remained viable at pH 5.5. Such a concentration is higher than that previously

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**FIG. 4.** Effect of fluoride inhibition of glycolysis during the adaptation period at pH 5.5 on survival of *S. mutans* UA159 at pH 3.5 for 30 min. Values represent the percent viable cells as shown by LIVE/DEAD BacLight staining and plating on blood agar. Symbols: ■, pH 5.5; □, pH 5.5/3.5; △, 0.5 M NaF; ▼, pH 5.5; □, 0.5 M NaF, pH 5.5/3.5.
employed with planktonic cells of *S. mutans* (1, 9, 13, 17); however, it is closer to the concentration of fluoride (3.15 M) in solutions and varnishes used clinically (26).

Comparisons between the different *S. mutans* strains in this study revealed that both planktonic and biofilm cultures of UA159 were more acid tolerant and induced a slightly stronger ATR than either H7 or XAB5. Such differences in acid tolerance between strains of the organism have been reported previously for studies with planktonic cultures (30); thus, it is clear that not all strains are the same. The development of dental caries is a gradual process caused by the emergence of an acid-tolerant microflora, with different individuals harboring different genotypes of *S. mutans* (21, 23, 25). Clearly, the genotypes that are capable of a rapid induction of an ATR will survive better in acidic plaque environments and constitute a higher risk for the development of caries.

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