Effect of acid stress on the physiology of biofilm cells of *Streptococcus mutans*

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*Streptococcus mutans* is a component of the dental plaque biofilm and an important aetiopathogenic agent in dental caries. Although this organism growing in the suspended (planktonic) state has been well characterized, relatively little is known about its physiology in biofilms, particularly in the acidic environments associated with caries development. The authors determined the effect of biofilm age (1–5 days) and cell density on selected metabolic properties under conditions of glucose limitation in a biofilm-chemostat at pH 7.5 and compared these baseline values with those of 3 day biofilms subjected to acid stress. Biofilm cell biomass more than doubled over the 5 day experimental period under baseline conditions, with the glycolytic rate, glucose uptake, glucose-PTS (phosphotransferase system) activity and protein synthesis maximum at 1–2 days. DNA and RNA synthesis increased for the first 3 days before decreasing in the 5 day biofilms, while H⁺/ATPase activity was higher in 5 day biofilms than 1 day biofilms, with overall activity 5–13-fold higher per cell unit than in the associated planktonic cells. Glucose pulsing (50 mM final concentration) for three consecutive days without pH control for 5 h (pH 4.39 ± 0.02) resulted in a progressive decrease in planktonic cell numbers; however, the rate of acid formation and glucose utilization in the chemostat by these cells increased per cell unit. Assays for carbohydrate metabolism in the latter cells showed increased activity, as did an assay for H⁺/ATPase (8-fold); however, DNA, RNA and protein synthesis were repressed (0.3–0.7-fold). Although the 3 day biofilm viable cell counts declined by 51% on glucose pulsing, all the physiological parameters measured by cell unit increased in activity, with notable increases in RNA and protein synthesis (4.6–7.6-fold). The results indicate that the maintenance of intracellular pH homeostasis is the basis of the enhanced physiological status and acid tolerance of biofilm cells.

**INTRODUCTION**

*Streptococcus mutans* is an important component of the biofilms on teeth (dental plaque) associated with many forms of dental caries (Bowden, 1991; Loesche, 1986). The organism rapidly metabolizes dietary carbohydrates, resulting in the formation of acid endproducts that can contribute to the demineralization of tooth enamel during caries development. *In vivo* pH telemetry has demonstrated that, depending on the age and composition of the plaque biofilm, and the concentration of the dietary carbohydrate, sugar consumption can result in the fluctuation of human plaque pH from 7 to 4 in as little as 3 min (Yamada et al., 1980). These daily cycles of acid shock are exacerbated by the prolonged consumption of sugars, leading to progressively lower plaque pH values between meals and during periods of sleep. The early studies of Stephan (1944) demonstrated that these ‘resting’ pH values decreased with increasing caries activity such that the plaque pH of individuals with severe caries showed consistently low pH values (≤5.5). Thus, organisms associated with caries not only contribute to the acidification of the plaque biofilm during the daily consumption of carbohydrate, but they must also be capable of growth and survival during the prolonged periods of low pH typical of caries development. With *S. mutans*, the exposure of exponential-phase planktonic cells to sublethal pH values (5.5–5.0) induces an acid tolerance response (ATR) that enhances cell growth and survival at lethal pH values (3.5–3.0) (Svensäter et al., 1997). More recently, biofilm cells of this organism in a biofilm-chemostat were shown to be significantly more acid tolerant than the planktonic cells in the same chemostat and they induced an ATR, albeit at a slower rate than that of exponential-phase batch-grown cells (McNeill & Hamilton, 2003).

The enhanced resistance of biofilm cells to adverse environmental conditions and antimicrobial agents, compared to cells grown in liquid culture, has been well established and has led to the concept that surface-associated cells are physiologically distinct from their planktonically grown counterparts (Costerton et al., 1987; Fletcher, 1991; Hoyle 

**Abbreviations:** ATR, acid tolerance response; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; RIC, rod incubation chamber.
Costerton, 1991; Brown & Gilbert, 1993; Goodman & Marshall, 1995; Davey & O'Toole, 2000). Recent research has demonstrated the biofilm-dependent regulation of gene expression and cell-to-cell signalling, including a variety of pathways invoked not only during the initiation of biofilm formation, but also during the succession of the biofilm community (O'Toole & Kolter, 1998; Christensen et al., 1998; Prigent-Combaret et al., 1999; Davey & O'Toole, 2000; Sauer et al., 2002). The adherence of oral streptococci to surfaces also results in complex changes in cell physiology, with quorum-sensing mechanisms playing a central role in the characteristics of the resulting biofilms (Loo et al., 2000; Li et al., 2001a). Biofilm cell density has been shown to modulate adaptation to acid tolerance at low pH, such that high cell density biofilms were more resistant to lethal pH values than those with lower cell densities (Li et al., 2001b).

Using a proteomics approach, protein expression in 3 day biofilm cells of S. mutans H7, growing in a biofilm-chemostat with limiting glucose at pH 7-5, was shown to be significantly different from that of the planktonic cells associated with the biofilms in the same chemostat (Svensäter et al., 2001). Of the proteins identified, 13 were unique to biofilm cells, while 9 were only expressed in planktonic cells. Overall, 20% of the detectable proteins were differentially expressed in biofilm cells compared to planktonic cells, confirming the concept that cells assuming growth on a surface undergo significant changes in physiology. More recently, biofilm cells of the organism were shown to be significantly more acid tolerant than planktonic cells, with glycolytic enzymes in the surface-associated cells up-regulated following a pH change from 7-5 to 5-5, suggesting that their enhanced acid resistance is associated with the maintenance of pH homeostasis (Welin et al., 2003).

In this study, we were interested in assessing selected physiological properties of biofilm cells of S. mutans BM71 growing over a 3 day period with limiting glucose in a biofilm-chemostat at a constant pH (7-5) in comparison with those properties in associated planktonic cells. Furthermore, to assess the effect of an acid shock typical of natural dental plaque biofilms, physiological tests were also carried out with biofilms subjected to three consecutive days of glucose pulses without pH control that resulted in pH decreases below 5-0 over a 5 h period.

METHODS

**Bacterial strain and media.** S. mutans BM71 was isolated from a caries lesion in a child (Bowden, 1991) and maintained anaerobically in complex liquid medium containing (per litre): tryptone (10 g), yeast extract (5 g) and 20 mM glucose buffered at pH 7-5 with 20 mM sodium/potassium phosphate buffer. Growth in the biofilm-chemostat was carried out anaerobically in the semi-defined medium of Bowden (1999) (MADM), which included 0-2 % tryptone and hog gastric mucin (0-025%; Sigma type 111), with 20 mM phosphate buffer and 20 mM glucose. To limit the planktonic cell concentration, this medium was used at one-quarter strength for all chemostat studies with the mean glucose concentration at 4-24 ± 0-15 mM. Rinse medium (RM) was MADM devoid of glucose and buffer, while dilution medium (DM) was MADM without glucose.

**Biofilm-chemostat.** Biofilms of S. mutans BM71 were generated in the controlled environment, rod-chemostat system of Bowden (1999) by the general methods previously reported (Li & Bowden, 1994a). Continuous baseline planktonic growth was achieved in the diluted MADM at pH 7-5 with a dilution rate (D) of 0-1 h⁻¹ (doubling time, t₀, 7 h) with glucose limitation. The gas phase was 5% CO₂ in nitrogen and the planktonic cultures were grown for at least 10 mean generations before steady state was considered to have been established. For biofilm formation, sterile epon hydroxypatite rods (Li & Bowden, 1994b) were aseptically inserted into either an 8- or 10-rand chemostat head and immersed in steady-state planktonic cultures for periods of 1-5 days. The growth area on each rod was 3-77 cm². To maintain consistency of biofilm growth, the same rods were inserted in the same locations in the chemostat head. Chemostats were monitored daily for cell density (Klett readings), pH and medium flow (dilution rate), with periodic samples removed for planktonic cell counts and glucose determinations.

**Bacterial sampling and viability.** For biofilm cell viability, the rods were removed from the chemostat and rinsed gently in DM prior to the removal of the cells by sonication for 60 s (Li & Bowden, 1994a). Viable cell counts (n=40) were determined by diluting and plating aliquots of the suspension on blood agar in triplicate as previously described (Svensäter et al., 1997). In addition, during baseline characterization the percentage live cells was determined using the Live/Dead Bacterial Viability method (LIVE/DEAD BacLight, Kit L-7012, Molecular Probes) and averaging the live and dead cell counts in five separate fields (McNeill & Hamilton, 2003). Planktonic cells were collected from the chemostat sample port and washed twice in DM before aliquots were diluted for counts and assessment of live and dead cells. The results are reported as the means±standard error for a minimum of three separate experiments involving a minimum of 24 rods.

**Glucose-pulse experiments.** To assess the effect of acid stress on the physiology of biofilm and planktonic cells, a steady-state chemostat culture was pulsed once a day (9 a.m.) with glucose (50 mM final concentration) for three consecutive days. Immediately before the initial glucose addition, sterile rods were inserted in the chemostat and left there for the 3 day pulse period (3 day biofilms). Immediately before the daily glucose addition, the pH control system was shut off and the pH of the culture was allowed to fall for 5 h before pH control was re-established at pH 7-5. For characterization of the glucose pulsing over the 3 day period, samples were removed daily from the planktonic phase just before the glucose addition and throughout the subsequent 12 h period, to measure culture pH, viable cell counts and the glucose concentration. The planktonic and biofilm cell samples used for metabolic analyses were removed at 9 a.m. on day 4, such that the cells had been exposed to medium at pH 7-5 for 19 h following the third and last glucose pulse. Glucose limitation of the planktonic culture was re-established within 12 h of the initiation of glucose pulsing. All such samples were subjected to dilution and plating to determine the viable cell counts.

**Rod incubation chamber.** Metabolic activity of intact cells in biofilms was carried out in a rod incubation chamber (RIC), which consisted of an acrylic cylinder (9 cm high × 1-8 cm in diameter) cemented to a flat base with a sampling port, fitted with a serum stopper, located 2 cm from the bottom of the cylinder. The rods were held in the chamber in a nylon cap capable of holding 5 rods. The RICs were located in a 37 °C incubator on a magnetic stirrer, with the medium mixed by a small stirring bar. With the rods inserted, the maximum medium volume was 22 ml, which allowed the removal of a total of 10 ml of medium for analysis with the rods.
still submerged in the liquid phase. In all cases, the assay medium was added to the RIC and pre-incubated at 37 °C before the rods were removed from the chemostat, gently rinsed in pre-warmed medium, and added to the chamber without disturbing the biofilms.

**Glycolytic rate.** The rate of acid formation from glucose was determined with cells in intact biofilms, with dispersed biofilm cells and with planktonic cells. The incubation medium (IB) was sterile 5 mM sodium/potassium phosphate buffer, pH 7.5, with 10 mM glucose as the carbon source. All pH titrations were carried out at 37 °C with a Radiometer Autoburette (model ABU 1a) (Hamilton & Buckley, 1991) and included five titrations of IB prior to each incubation (pre-zero time) to establish the baseline volumes of standardized KOH required to reach pH 7.50. Intact biofilms and cells dispersed from biofilms were assayed on the same day by using five alternate rods for the respective assays. Intact biofilms were gently rinsed in 2.0 ml sterile, pre-warmed buffer and then immediately (zero time) added to the RIC containing 22 ml IB. At 30, 60, 90 and 120 min, 3.0 ml samples were removed from the RIC by a sterile syringe, added to the pH stat reaction chamber and the amount of KOH required to titrate to 7.50 recorded. Following removal of the final sample, the rods were rinsed in RM, and the cells were removed by sonication and plated on blood agar as described previously. To correct for the contribution of shed cells to the overall acid production, the remaining medium in the RIC following the last sample was centrifuged and the cell count determined. The net acid formation was determined at each time point and the results reported as nmol acid formed min⁻¹ (10⁶ cells)⁻¹, with the values corrected for the acid generated by the cells lost from the rods (2.8±0.7% of the total). Each daily assay consisted of one set of five rods, with the results of at least three such daily measurements used for 1, 2, 3 and 5 day biofilms.

For the dispersed biofilm cells, the rods were gently rinsed in 2.0 ml cold, sterile 5 mM buffer and the cells removed from the rods by sonication into the same buffer. The dispersed biofilm suspensions containing 15 ml of the same buffer and 300 μl immediately (time zero) added to a RIC incubated at 37 °C in a pH stat reaction vessel containing a small stirring bar. At time zero, 40 μl [¹⁴C]glucose was added, and 0.5 ml samples were removed at 10, 20 and 30 s and added to 0.5 ml 30 mM NaF to stop the reaction. The cells were filtered, washed and their radioactivity measured as described above. The results are reported as nmol glucose taken up min⁻¹ (10⁶ cells)⁻¹.

**Macromolecular synthesis.** DNA, RNA and protein synthesis by 1, 2, 3 and 5 day biofilms was assayed by the respective uptake of [¹⁴H]thymidine, [³H]uridine and a mixture of [¹⁴C]-labelled amino acids (Hilliard et al., 1999). Each assay consisted of duplicate rods removed from the chemostat, rinsed gently in MADM medium and immediately immersed in a RIC containing 15 ml pre-warmed MADM growth medium plus 150 μl of the appropriate substrate and incubated at 37 °C for 5 min. DNA synthesis was measured with [methyl-³H]thymidine (70 Ci mm⁻¹ at 0.5 μCi ml⁻¹; Amersham), RNA synthesis with [³H]uridine (25 Ci mm⁻¹ at 0.1 μCi ml⁻¹; Amersham) and protein synthesis with a [¹⁴C]-labelled amino acid mixture (54 mCi atom carbon⁻¹ at 1.0 μCi ml⁻¹; NEN) [1 Ci = 37 GBq]. At 5 min, the rods were removed and added to 2.0 ml cold sterile DM and the cells sonicated from the rods. Triplicate 10 μl samples were removed for plate counts and this was followed immediately by the addition of 2.0 ml cold 10% TCA to the remaining cells. This suspension was filtered through glass microfibre filters (Whatman), washed with 5.0 ml cold 10% TCA and finally with 5.0 ml cold distilled water. The filters were then dried and their radioactivity measured in 4 ml Aquasol. Planktonic cells were collected from the chemostat sample port, washed, resuspended in MADM, and samples diluted for plate counting as previously described. The reactions were initiated by the addition of 10 μl of the radioactive substrate to tubes containing 1.0 ml pre-warmed cell suspension. For the uptake of [¹⁴H]thymidine and [³H]uridine, the reactions in duplicate tubes were terminated at 2 and 5 min by the addition of 10% TCA followed by filtration as described above. A similar method was used to measure the uptake of [¹⁴C]-labelled amino acids with the exception that 100 μl cold Casamino acids (5 mg ml⁻¹) was added with the 10% TCA at the termination of uptake at 2 and 5 min. The results are expressed as radioactivity taken up by cells: pmol min⁻¹ (10⁶ cells)⁻¹.

**ATPase and glucose-PTS activity.** H⁺/ATPase activity was assayed in permeabilized cells by measuring the release of inorganic phosphate from ATP (Belli & Marquis, 1994), while glucose-PTS was assayed in permeabilized cells by the phosphoenolpyruvate (PEP)-dependent uptake of [¹⁴C]glucose via the phosphotransferase system (PTS) (Hamilton et al., 1989). Cells, sonicated from a minimum of 10 rinsed biofilm rods, were pooled in 2.0 ml DM and 10 μl samples removed for cell counts. Planktonic cells from the same chemostat were collected as described above. Both biofilm and planktonic cells were then washed twice, resuspended in cold, sterile permeabilization buffer (75 mM Tris/HCl (pH 7.0), 10 mM MgCl₂) before being frozen in 1.0 ml aliquots. Permeabilization was carried out immediately prior to each assay and involved thawing a 1-0 ml suspension, adding 50 μl of a toluene/acetone mixture (1:9) and vigorously mixing for 5 min with cooling in ice every 60 s. For the ATPase assay, cells were washed twice and resuspended in assay buffer consisting of 50 mM Tris/maleate buffer (pH 6.0) and 20 mM MgCl₂. Triplicate reactions (0.5 ml) were initiated by the addition of ATP (5 mM, final concentration) with incubation at 37 °C for 30 min. Controls included cells without ATP and ATP without cells. The reactions were stopped by the addition of 25 μl radioactivity measured in a liquid scintillation counter (Beckman). A minimum of six biofilms were assayed for each age of biofilm. Planktonic cells were collected as described above, washed and resuspended in 7.5 ml sterile 50 mM buffer; triplicate 10 μl samples were diluted and plated for cell counts. Triplicate uptake reactions consisted of 2-0 ml cell suspension equilibrated at 37 °C in a pH stat reaction vessel containing a small stirring bar. At time zero, 40 μl [¹⁴C]glucose was added, and 0.5 ml samples were removed at 10, 20 and 30 s and added to 0.5 ml 30 mM NaF to stop the reaction. The cells were filtered, washed and their radioactivity measured as described above. The results are reported as nmol glucose taken up min⁻¹ (10⁶ cells)⁻¹.
2 M HCl and the phosphate released was measured by the Fiske-SubbaRow method (Sigma Diagnostics). The results were expressed as nmol P<sub>i</sub> released min<sup>−1</sup> (10<sup>9</sup> cells)<sup>−1</sup>. For the glucose-PTS assay, cells were washed twice and resuspended in assay buffer consisting of 50 mM phosphate buffer (pH 7.5), 4 mM MgCl<sub>2</sub>, 20 mM NaF and 5 mM 2-mercaptoethanol. Triplicate assays (0.5 ml) of pre-warmed cell suspension plus 4 mM PEP were initiated by the addition of 10 µl [¹⁴C]glucose (1·0 µCi ml<sup>−1</sup>) with incubation at 37°C for 15 min. Controls included cells and [¹⁴C]glucose without PEP. The reactions were stopped by the addition of 4·5 ml ethanolic BaBr<sub>2</sub> and the suspensions filtered through Whatman HA filters (0·45 µm), following a 20 min incubation in ice, and counted in Aquasol as described above. The results are reported as nmol glucose taken up min<sup>−1</sup> (10<sup>9</sup> cells)<sup>−1</sup>.

**Analyses.** The glucose concentration in the medium was determined with glucose oxidase by the method of Kingsley & Getchell (1960). Statistical analysis was carried out with the computer program Prism 4 for Macintosh.

**RESULTS**

**Cell growth and viability**

The baseline growth conditions in the biofilm-chemostat involved the anaerobic growth of *S. mutans* BM71 at pH 7·5 in a diluted semi-defined medium (MADM) with glucose limitation at a dilution rate of 0·1 h<sup>−1</sup> (t<sub>0</sub> = 7 h) for the planktonic cells. These conditions generated 615 × 10<sup>6</sup> ml<sup>−1</sup> of viable steady-state planktonic cells as estimated by plate counts on blood agar, which was shown by assay with the Live/Dead fluorescent staining assay to be 76·2 % of the total cells. Under steady-state conditions, the biofilms formed on epon hydroxyapatite rods increased in cell concentration over time, with 5 day biofilms twice the cell biomass of the 1 day biofilms: 11·2 ± 1·2 × 10<sup>6</sup> to 22·3 ± 1·0 × 10<sup>6</sup> cells cm<sup>−2</sup>, respectively. Significant differences were observed between days 1–2 (15·2 ± 0·9), 1–3 (19·6 ± 0·8) and 1–5 (P < 0·05). Of particular interest was the observation that the mean percentage of live cells in the four biofilms assayed over the 5 day period (78·0 %) was similar to that of the planktonic cells, confirming previous observations (McNeill & Hamilton, 2003).

**Baseline physiological status**

The physiology of biofilm cells was assessed under two sets of conditions: during growth with limiting glucose at pH 7·5 (baseline conditions) and following a period of acid stress that resulted from three daily glucose pulses, each followed by a 5 h period without pH control. The growth at pH 7·5 provided the baseline physiological status of 1–5 day-old biofilms as a control for the comparisons made between 3 day baseline and glucose-pulsed biofilms. Planktonic cells generated in the chemostat under the same conditions were also included in all assays to provide a comparison to their associated biofilms.

Carbohydrate metabolism, as estimated by the glycolytic rate of biofilm cells, was assessed with intact biofilms and with dispersed cells removed from the biofilms. As seen in Fig. 1, baseline acid formation by intact biofilms decreased with age, with 1 day biofilms 14-fold more active than 5 day biofilms [638 ± 60 vs 46 ± 10 nmol acid formed min<sup>−1</sup> (10<sup>9</sup> cells)<sup>−1</sup> (P < 0·05)]. The results were corrected for the number of cells shed during the assay, which was minimal [2·8 (± 0·7) %]. Interestingly, the activity of the biofilm cells removed from alternate rods was higher than that of the intact biofilms, ranging from 2·1 to 4·7-fold higher for the 1 and 5 day biofilm cells, respectively. In addition, the loss in activity over the 5 day period was only 6-fold [1315 ± 163 (1 day) vs 212 ± 55 (5 days) nmol acid formed min<sup>−1</sup> (10<sup>9</sup> cells)<sup>−1</sup>]. The glycolytic rate of the planktonic (P) cells associated with the biofilms was 295 nmol acid formed min<sup>−1</sup> (10<sup>9</sup> cells)<sup>−1</sup>, less than that of the 1 and 2 day intact biofilms.

To complement the glycolytic data, glucose uptake measurements were carried out with intact biofilm cells over the 1–5 day period, as well as assays for glucose phosphorylation via the glucose-PTS with decryptified biofilm cells for the same period. As seen in Table 1, glucose uptake by intact biofilms was maximum for 2 day biofilms, with uptake decreasing 4-fold for the 5 day biofilms. Similarly, glucose-PTS
activity was also the highest with the 2 day biofilms, decreasing 3-fold for the 3- and 5 day biofilms. Since glycolytic activity and intracellular pH homeostasis are intimately associated in S. mutans (Bender et al., 1986; Hamilton & Buckley, 1991), the level of activity of the proton-pumping H+/ATPase was also assayed with decrptified biofilm cells. The 2 day biofilm cells had the highest activity; however, the 5 day biofilm cells still possessed almost 2-fold higher activity than the 1 day cells, suggesting that H+ removal from the cell cytoplasm was maintained in the older cells. Comparative activity measurements for the planktonic cells in the same chemostats (see Table 4) showed that glucose uptake activity was more than 2-fold higher than in the 2 day biofilms (Table 1), while glucose-PTS activity was significantly lower (25-fold) than that of these biofilm cells (P < 0.06). Furthermore, the biofilm cells possessed 5- to 13-fold higher H+ /ATPase activity per cell unit than planktonic cells under the same conditions (P < 0.04).

The status of macromolecular synthesis in biofilm cells of S. mutans H7 over the 5 day period was assessed by the incorporation of [3H]thymidine into DNA, [3H]uridine into RNA and 14C-labelled amino acids into protein. As seen in Fig. 2, the uptake of [3H]thymidine and particularly [3H]uridine increased over the first 3 days before decreasing in the 5 day biofilm cells. Incorporation of 14C-labelled amino acids, on the other hand, was highest in the 1 day biofilm cells and decreased with age such that the 5 day biofilm cell results were 3-fold lower than those of the 1 day cells. Biosynthetic activity by the planktonic cells from the same chemostats (Table 4) was higher than that of the associated 3 day biofilms (Fig. 2), with significant differences observed for uptake of 14C-labelled amino acids (16-4-fold; P < 0.06) and [3H]thymidine (8-4-fold; P < 0.06), while [3H]uridine incorporation was 2-3-fold higher, indicating the more active growth status of the planktonic cells.

Physiological changes following glucose pulse
To test the effect of acid stress on the physiology of biofilm and planktonic cells of S. mutans BM71, a glucose pulse (50 mM, final concentration) was applied to each chemostat once a day for three consecutive days with the 5 h period following the pulse carried out in the absence of pH control. As seen in Table 2, the terminal pH of the planktonic culture at the fifth hour on each of the days was similar (mean pH 4.39 ± 0.02); however, the percentage of planktonic cell counts that survived the low minimum pH decreased by 80% from day 1 to day 3, indicating that the cells were stressed by the acidic conditions. This pH reduction was, however, accomplished by fewer cells, as indicated by the significant increase in the initial rate of the acid production from 0.98 to 5.23 μM H+ h⁻¹ between day 1 and day 3. Furthermore, the rate of glucose utilization per unit cell biomass increased almost 3-fold over this same period [327 to 850 nmol (10⁶ cells)⁻¹], confirming the adaptation of planktonic cells of S. mutans to increased glycolytic activity in response to periodic applications of high concentrations of sugar (Bradshaw et al., 1989).

The mean biofilm cell count following the 3 days of glucose pulses was 9.4 ± 1.19 × 10⁶ cm⁻², representing a 50-8% reduction in cell numbers compared to the baseline counts. Nevertheless, the results for 3 day biofilms (Table 3) showed that glucose pulsing and the resultant acidification of the liquid phase associated with the biofilms enhanced the activity of all of the measured physiological parameters. With respect to carbohydrate metabolism and transport, the glycolytic rate increased 6-1-fold, while overall glucose transport and glucose-PTS activity were enhanced 2-4- and 7-2-fold, respectively. While not increased to the same degree as the planktonic cells, the biofilm H+ /ATPase activity was, nevertheless, 2-fold higher after glucose pulsing and the resultant acidification of the medium. Unlike the planktonic cells, 3 day biofilms had higher levels of

### Table 2. Effect of three daily glucose pulses (50 mM, final concentration) on the culture pH, cell counts, acid formation and glucose utilization by planktonic cells of S. mutans BM71 in a biofilm-chemostat (D=0.1 h⁻¹) following a 5 h period without pH control

<table>
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<tr>
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<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<tr>
<td><strong>Culture pH</strong></td>
<td></td>
<td></td>
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<tr>
<td>At 0 h</td>
<td>7.30</td>
<td>7.30</td>
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<tr>
<td>At 5 h</td>
<td>4.40</td>
<td>4.40</td>
<td>4.37</td>
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<tr>
<td><strong>Cell counts (10⁶ ml⁻¹)</strong></td>
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<tr>
<td>At 0 h</td>
<td>186</td>
<td>161</td>
<td>195</td>
</tr>
<tr>
<td>At 5 h</td>
<td>81.9</td>
<td>49.6</td>
<td>18.4</td>
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<tr>
<td>Percentage of cells at 5 h</td>
<td>44.0</td>
<td>30.8</td>
<td>9.4</td>
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<tr>
<td><strong>Acid formation (μM H+ h⁻¹)</strong></td>
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<tr>
<td>Initial rate</td>
<td>0.98</td>
<td>3.43</td>
<td>5.23</td>
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<tr>
<td><strong>Glucose utilization [nmol (10⁶ cells)⁻¹]</strong></td>
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<td></td>
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<tr>
<td>At 2 h</td>
<td>41</td>
<td>50</td>
<td>59</td>
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<tr>
<td>At 5 h</td>
<td>327</td>
<td>664</td>
<td>850</td>
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biosynthesis, with uridine and amino acid uptake enhanced 4-6- and 7-6-fold respectively, while thymidine uptake was enhanced less than 2-fold.

As predicted from the glucose utilization data during growth in the chemostat, the 3 day glucose pulses had a profound effect on the overall physiological status of the planktonic cells. As seen in Table 4, the glycolytic rate and glucose uptake by cells subjected to 3 days of glucose pulses were 1-6- and 2-0-fold higher than the baseline values, while glucose-PTS activity was 43-fold higher ($P < 0.05$). The enhanced need for intracellular pH homeostasis in the glucose-pulsed cells was evident by a 7-9-fold increase in $H^+$/ATPase activity ($P < 0.05$). This enhanced activity contrasted with that of macromolecular synthesis: DNA, RNA and protein synthesis were adversely affected by acid stress, with glucose pulse/baseline ratios of 0-3, 0-7 and 0-3, respectively. Comparisons between biofilm (Table 3) and planktonic cells (Table 4) under baseline conditions revealed significantly lower glycolytic activity ($P < 0.06$) in biofilm cells, but significantly higher $H^+$/ATPase activity ($P < 0.04$). Interestingly, this latter activity was also significantly higher ($P < 0.06$) in biofilm cells following the 3 day pulsing period.

**DISCUSSION**

Previous studies with *S. mutans* BM71 in the biofilm-chemostat system employed in this study demonstrated the consistent formation of stable biofilms under conditions approximating those of the oral cavity in which a constant supply of mucin was added to simulate the presence of saliva, along with a limiting supply of glucose (Li & Bowden, 1994a). These conditions reflect those to which dental plaque biofilms are exposed during the majority of the day (~23 h) for individuals in Western societies eating a balanced diet and not consuming refined sugar frequently between meals (Watanabe & Dawes, 1988; Hamilton & Bowden, 2000). Cell counts obtained over 26 separate chemostat runs showed cell numbers doubling between days 1 and 5; however, the doubling time between days 3 and 5 indicated very slow growth (340 h). That such conditions were appropriate for the study of *S. mutans* physiology in a biofilm is reflected by the fact that the growth rates obtained by strain BM71 in the biofilm-chemostat were similar to those obtained for *S. mutans* in an animal model system (Beckers & van der Hoeven, 1982). Furthermore, the chemostat model is effective in standardizing the overall environmental growth conditions associated with the biofilms over the experimental period. However, one must be aware of the fact that while the planktonic cells associated with the biofilm may be in steady-state growth, this would not hold for the biofilm community itself. As biofilms are heterogeneous in nature (Wimpenny et al., 2000), the results are reported on the basis of colony-forming units and would represent the mean physiological activity of the biofilm.

Generally, physiological activity under baseline conditions was highest within the first 3 days and decreased with the 5 day rods as the growth rate declined. Predictably, activity of the associated steady-state planktonic cells under the baseline conditions was higher than that of the slower-growing biofilm cells, except for glucose-PTS and $H^+$/ATPase activity (Table 3 vs Table 4). The increased glycolytic activity upon dispersal of the cells from the rods (Fig. 1) would suggest glucose diffusion was limited in the intact biofilms, reducing glucose uptake. Although viable cell numbers on the rods were not high, ranging from 1-1 to 2-2 x 10^7 cm^-2 over the 5 day period, it is conceivable that cells inside small clusters in the biofilm would be deprived of glucose, causing a lower biofilm glycolytic rate compared to the same cells removed from the rods (Stewart, 2003). This concept is

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**Table 3.** Baseline cellular activity of 3 day biofilm cells of *S. mutans* BM71 growing in a biofilm-chemostat with glucose limitation at a constant pH of 7-5 in comparison to biofilm cells subjected to three daily glucose pulses without pH control.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Baseline (BL)</th>
<th>Glucose pulse (GP)</th>
<th>Ratio GP/BL</th>
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<tbody>
<tr>
<td>Glycolytic rate*</td>
<td>106 ± 11</td>
<td>649 ± 85</td>
<td>6-1</td>
</tr>
<tr>
<td>Glucose uptake†</td>
<td>395 ± 85</td>
<td>949 ± 57</td>
<td>2-4</td>
</tr>
<tr>
<td>Glucose-PTS‡</td>
<td>69 ± 20</td>
<td>20 ± 49</td>
<td>7-2</td>
</tr>
<tr>
<td>ATPase§</td>
<td>1063 ± 270</td>
<td>2117 ± 317</td>
<td>2-0</td>
</tr>
<tr>
<td>Thymidine uptake§</td>
<td>35-7 ± 8-7</td>
<td>97-1 ± 19-9</td>
<td>1-6</td>
</tr>
<tr>
<td>Uridine uptake§</td>
<td>120 ± 37</td>
<td>555 ± 22</td>
<td>4-6</td>
</tr>
<tr>
<td>Amino acid uptake§</td>
<td>9-8 ± 2-3</td>
<td>74-9 ± 11-6</td>
<td>7-6</td>
</tr>
</tbody>
</table>

*See Table 3 for units.

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**Table 4.** Baseline cellular activity of steady state planktonic cells of *S. mutans* BM71 growing in a biofilm-chemostat with glucose limitation at a constant pH of 7-5 in comparison to planktonic cells subjected to three daily glucose pulses without pH control.

<table>
<thead>
<tr>
<th>Assay*</th>
<th>Baseline (BL)</th>
<th>Glucose pulse (GP)</th>
<th>Ratio GP/BL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolytic rate</td>
<td>295 ± 75</td>
<td>487 ± 75</td>
<td>1-6</td>
</tr>
<tr>
<td>Glucose uptake</td>
<td>1191 ± 360</td>
<td>2403 ± 998</td>
<td>2-0</td>
</tr>
<tr>
<td>Glucose-PTS</td>
<td>10-3 ± 1-4</td>
<td>446 ± 35</td>
<td>43</td>
</tr>
<tr>
<td>ATPase</td>
<td>119 ± 23</td>
<td>938 ± 357</td>
<td>7-9</td>
</tr>
<tr>
<td>Thymidine uptake</td>
<td>300 ± 87</td>
<td>152 ± 18</td>
<td>0-5</td>
</tr>
<tr>
<td>Uridine uptake</td>
<td>280 ± 54</td>
<td>186 ± 39</td>
<td>0-7</td>
</tr>
<tr>
<td>Amino acid uptake</td>
<td>161 ± 22</td>
<td>46-8 ± 7-9</td>
<td>0-3</td>
</tr>
</tbody>
</table>

*See Table 3 for units.
supported by the fact that the ratio of dispersed/intact cell activity increased progressively with the increase in biofilm cell numbers over the 5 day period, e.g. 2.1, 2.5, 3.2 and 4.6 for the 1, 2, 3 and 5 day biofilms, respectively. *S. mutans* BM71 is known to form microcolonies readily on glass rods in the same biofilm-chemostat system (Li & Bowden, 1994a).

The pulsing of the chemostat with glucose in the absence of pH control was initiated to simulate the conditions that dental plaque can be subjected to during dietary consumption of refined sugars (Stephan, 1944; Hamilton, 2000). The concentration of glucose (50 mM) was chosen to provide a period of acid stress in order to test the physiological response of both biofilm and planktonic cells. That acid stress had been achieved was apparent from the fact that the pH minimum (4.37–4.40) observed over the 3 day pulse period (Table 2) was 0.40 pH units below the pH limit for growth of the organism (Hamilton, 1986), indicating uncoupling of growth and metabolism. This is reflected in the gradual decline in the planktonic viable cell counts over the 3 day pulse period from 44.0 to 9.4%. Whether the loss of cells from the planktonic phase during the glucose pulse period was due to acid-induced growth inhibition or killing is not known; however, such cells have a poor capacity to induce an ATR that would aid survival at low pH, suggesting inhibition of cellular biosynthesis triggered by cytoplasmic acidification (Hamilton & Buckley, 1991; McNeill & Hamilton, 2003). This is supported by the observation that thymidine, uridine and amino acid uptake into glucose-pulsed planktonic cells was inhibited compared to baseline cells. It should be noted that, although the cell counts declined during the 5 h glucose-pulsing period on each of the 3 days, the cell numbers increased to normal or pre-pulse values during the 19 h following the resumption of growth at pH 7.5, the last 12 h of which were under glucose-limited conditions.

The 3 days of glucose pulsing and concomitant acidification resulted in lower numbers of biofilm cells (51%) than on the rods under baseline conditions; nevertheless, pulsing did have a stimulatory effect on all of the measured physiological properties of the biofilm cells compared to that under baseline conditions (Table 3). The enhanced glucose uptake and metabolism by the biofilm cells during the 3 day pulsing period is indicative of ‘carbohydrate training’, which has been shown to be associated with the emergence and domination of the acidogenic/aciduric microflora in mixtures of oral bacteria (Bradshaw et al., 1989) and in dental plaque of human subjects (Stephan, 1944). Furthermore, the increased glycolytic activity is consistent with recent proteomic data showing that the synthesis of glycolytic enzymes in biofilm cells of *S. mutans* H7 is up-regulated during pH changes from 7.5 to 5.5 compared to control cells maintained at pH 7.5 (Welin et al., 2003). This was made possible since, unlike the associated planktonic cells, biofilm cells increased the uptake of thymidine, uridine and amino acids into DNA, RNA and protein (Table 3). These latter results are consistent with the enhanced growth and biofilm formation observed previously with *S. mutans* BM71 under conditions of glucose excess (Li & Bowden, 1994a, b) and concur with the recent observation that biofilm cells possessed a greater capacity to induce an ATR than the associated planktonic cells growing in the same biofilm-chemostat (McNeill & Hamilton, 2003).

Clearly, our results indicate that biofilm cells, under conditions of acid stress, were more physiologically ‘fit’ than the associated planktonic cells in the same chemostat. The key physiological trait would appear to be related to the enhanced capacity of biofilm cells to maintain cellular pH homeostasis. Intracellular pH levels in *S. mutans* are maintained above that of the external environment by proton extrusion via membrane-associated H+/ATPase (Bender et al., 1986) and lactate efflux (Dashper & Reynolds, 1996), and it is known that the sustained growth of the organism at pH 5.0–5.2 results in the increased synthesis of lactic dehydrogenase (Wilkins et al., 2002) and H+/ATPase (Hamilton & Buckley, 1991). This latter result with planktonic cells of *S. mutans* Ingbritt demonstrated that growth at progressively lower pH values was correlated to increases in H+/ATPase activity, a result confirmed with both the biofilm (Table 3) and planktonic (Table 4) cells of *S. mutans* BM71 in this study. The maintenance of H+/ATPase activity in the 3- and 5 day biofilm cells under baseline conditions in spite of declining glycolytic activity (Table 1, Fig. 1) suggests that sufficient ATP was being generated to drive H+ expulsion from the cell. Furthermore, the significantly higher level of ATPase activity in the baseline biofilm cells compared to that in the planktonic cells indicates that the biofilm environment may be more acidic than the bulk liquid phase, a situation which would account for the high level of ATPase activity in the baseline biofilm cells. The capacity to maintain cellular pH could explain the higher level of macromolecular synthesis during glucose pulsing by biofilm cells and account for the enhanced capacity of such cells to induce an ATR when subjected to a pH change from 7.5 to 5.5 (McNeill & Hamilton, 2003; Welin et al., 2003).

**ACKNOWLEDGEMENTS**

This study was supported by a grant to I.H. from the Canadian Institutes of Health Research (MT-3546).

**REFERENCES**


Bacterial resistance to 


