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Persistence of *Streptococcus mutans* in Stationary-Phase Batch Cultures and Biofilms†

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Streptococcus mutans **is a member of oral plaque biofilms and is considered the major etiological agent of dental caries. We have characterized the survival of *S. mutans* strain UA159 in both batch cultures and biofilms. Bacteria grown in batch cultures in a chemically defined medium, FMC, containing an excess of glucose or sucrose caused the pH to decrease to 4.0 at the entry into stationary phase, and they survived for about 3 days. Survival was extended up to 11 days when the medium contained a limiting concentration of glucose or sucrose that was depleted by the time the bacteria reached stationary phase. Sugar-limited cultures maintained a pH of 7.0 throughout stationary phase. Their survival was shortened to 3 days by the addition of exogenous lactic acid at the entry into stationary phase. Sugar starvation did not lead to comparable survival in biofilms. Although the pH remained at 7.0, bacteria could no longer be cultured from biofilms 4 days after the imposition of glucose or sucrose starvation; *Bac*Light staining results did not agree with survival results based on culturability. In both batch cultures and biofilms, survival could be extended by the addition of 0.5% mucin to the medium. Batch survival increased to an average of 26 (±8) days, and an average of 2.7×10^5 CFU per chamber were still present in biofilms that were starved of sucrose for 12 days.**

Streptococcus mutans is normally found within the oral cavity as a member of the dental plaque community. Even though dental plaque consists of numerous bacterial species, only the presence of *S. mutans* has been consistently linked with the production of dental caries (18). Within the oral cavity, *S. mutans* is exposed to feast or famine conditions with regard to dietary sugars. The presence of excess sugar allows for bacterial growth and the production of lactic acid. Subsequently, between sugar pulses, *S. mutans* may persist in a sugar-starved environment. Sugar starvation has been shown to induce stationary-phase survival in batch cultures of gram-positive bacteria such as *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus pyogenes* (4, 6, 9, 29, 30, 36, 40). *S. mutans* is naturally a biofilm-forming bacterium (2), and we have characterized its survival in biofilms as well as in batch cultures.

A large amount of work has been done to identify genes that are required for the proper formation of biofilms by *S. mutans* (1, 7, 8, 13, 16, 20, 22, 25, 41, 42, 43). However, little is known about the survival of *S. mutans* within mature biofilms. Studies of the survival of *S. mutans* have focused primarily on its response to periods of acid shock. It has been shown that *S. mutans* grown in both batch cultures and biofilms can develop an acid tolerance response when exposed to a sublethal decrease in pH to 5.5 (14, 21, 32). Carbon starvation has been shown to enhance the acid tolerance of bacteria grown in both batch cultures and biofilms (31, 33, 45), but the survival of carbon-starved *S. mutans*, independent of an acid shock, has not been studied.

For this study, the survival of *S. mutans* UA159 was characterized in a chemically defined medium, FMC (34). Bacteria survived less well under conditions of sugar excess than of sugar limitation. The loss of viability with excess sugar was associated with a decrease in the pH of the culture. Similar effects were observed with glucose or sucrose as a carbon source. Bacteria in a monospecies biofilm survived for only 3 days in the absence of sugar. The duration of survival was extended by the addition of mucin, a major glycoprotein found within human saliva, for both planktonic and biofilm bacteria that were starved for sugar. *Bac*Light staining, which measures the presence of intact membranes, did not correlate with the culturability of bacteria from biofilms. Biofilms treated with 10% formalin for 1 h retained intact membranes according to *Bac*Light staining, suggesting that this stain may not be a good indicator of *S. mutans* viability in biofilms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strain used for all experiments was *S. mutans* UA159. The strain was stored in 30% glycerol and revived in Todd-Hewitt broth (THB) (Difco, Detroit, Mich.) or FMC (34) supplemented with 24 mM glucose in a 5% CO_2 incubator at 37°C overnight prior to use in experiments. FMC (34) was prepared without glucose and cysteine. FMC or THB was supplemented with various concentrations of glucose or sucrose. FMC or THB was supplemented with 100 mM glucose or sucrose to achieve sugar-excess conditions. THB contains approximately 10 mM glucose, and thus the addition of 100 mM glucose brought the final concentration to 110 mM. FMC was supplemented with 6 mM glucose or 3 mM sucrose to achieve sugar-limiting conditions. The biofilm starvation medium was fresh FMC with no sugar. A 5% stock solution of type III partially purified pig gastric mucin (Sigma, St. Louis, Mo.) was prepared as previously described (38) and added to the culture medium at 2, 1, or 0.5%.

Batch culture growth and survival. Overnight cultures grown in FMC with 24 mM glucose were diluted 25-fold into fresh FMC containing limiting or excess sugar. Cultures grown in THB were diluted 25-fold into fresh THB with or without additional glucose (100 mM). *S. mutans* UA159 was grown in culture tubes exposed to air at 37°C, and growth was monitored by use of a Spectronic Genesys 5 spectrophotometer (Milton Roy, Ivyland, Pa.) to measure the optical density at 675 nm (OD_{675}). Cultures grown in FMC containing glucose were

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† B.A.B. dedicates this article to Peter J. Buttaro (1930–2004).

tested for the presence of glucose after entry into stationary phase by use of a glucose-peroxidase kit as described by the manufacturer (Sigma). The culture pH was determined with pH indicator paper (Whatman, Maidstone, United Kingdom). For determinations of survival, samples were removed, serial dilutions of each culture were made in 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) or 154 mM NaCl, and duplicate samples were plated onto TH agar.

Survival in batch culture was also monitored by using the Live/Dead *Bac*Light viability stain as described by the manufacturer (Molecular Probes, Eugene, Oreg.). Imaging was performed with a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, N.Y.) with fluorescein isothiocyanate and rhodamine filters. Images were viewed with a 100× oil Plan Fluor objective and captured with Image-Pro Plus (Mediacybernetics, Carlsbad, Calif.). Adobe Photoshop 7.0 was used to overlay SYTO 9 and PI images.

The effect of a fall in pH was tested by artificially lowering the culture pH to 4.0 by the addition of lactic acid (80 mM) or HCl (80 mM) as the cultures entered stationary phase.

Biofilm formation. Biofilms were grown in flow cell chambers (27) exposed to air at 37°C. FMC containing 3 mM sucrose or 6 mM glucose was supplied at a flow rate of 200 μl min^{-1}. The assembly of the flow cell system was described previously (27). *S. mutans* UA159 was grown overnight in batch cultures in FMC containing 24 mM glucose in a 5% CO_2 incubator at 37°C. The bacteria were diluted 25-fold into fresh FMC containing 24 mM glucose and were allowed to grow for 4 to 5 h. The bacteria were washed twice with PBS or 154 mM NaCl and then diluted to an OD_{675} of 0.1. Five hundred microliters of the diluted culture was injected directly into the flow chamber tubing by use of a syringe. Immediately after all chambers were inoculated, the flow (200 μl min^{-1}) was started with an Ismatec (Glattbrugg, Switzerland) digital pump. The chambers were initially inverted for 20 min to allow the bacteria to adhere to the glass coverslip, and then the chambers were returned to an upright position. Up to eight chambers were processed in parallel. After the growth period, the biofilms were starved by replacing the growth medium with FMC containing no sugar but supplemented with mucin when indicated.

Biofilm imaging and survival. Biofilms were monitored by use of the *Bac*Light stain. Three microliters of the stock solution was diluted into 1 ml of PBS. A 500-μl volume of the diluted stain was injected directly into the flow cell tubing. The flow cell chamber was incubated at room temperature for 15 min and viewed with a FluoView 300 confocal scanning laser microscope (Olympus America Inc.; Melville, N.Y.) with a 60× PLAPO oil objective. Images were captured with Olympus FluoView imaging software.

To monitor biofilm survival, we removed the glass coverslip from the biofilm chamber with a sterile razor blade. The biofilm was then scraped from the glass coverslip by use of a sterile surgical blade and was resuspended in 1 ml of reduced transport fluid (RTF) (19). The suspension was vortexed, and 100 μl was spread onto TH agar. The remaining suspension was sonicated by use of a cell disrupter (Heat Systems model 185; Ultrasonics Inc., Plainview, N.Y.) with a microtip for 20 s (setting 4). The suspension was serially diluted in RTF and plated on TH agar, unless otherwise indicated. The results were recorded in CFU per chamber.

RESULTS

Conditions for stationary-phase survival in batch culture. *S. mutans* UA159 grown in FMC containing 100 mM glucose or sucrose reached an average OD_{675} of 1.63 or 1.29, respectively, at the entry into stationary phase. The lower value obtained for sucrose cultures was thought to result from the clumping of the bacteria because of the production of extracellular glucans. When FMC contained either 6 mM glucose or 3 mM sucrose, the cultures entered stationary phase at an average OD_{675} of 0.57 or 0.65, respectively. Cultures grown in FMC containing 6 mM glucose were determined to have exhausted the glucose at the entry into stationary phase by a glucose-peroxidase test. Bacteria grown in the presence of 100 mM glucose entered stationary phase with glucose still present in the medium. Stationary-phase survival was monitored by colony formation on TH agar (Fig. 1). Cultures were considered to contain no viable bacteria when plating yielded no colonies on TH agar (<10 CFU ml^{-1}). In the presence of excess glucose, no colonies were obtained from cultures that had been in stationary phase for 3 days. However, glucose-starved bacteria survived for up to 11 days in stationary phase (Fig. 1). In glucose-starved cultures, an initial drop in the viable count occurred 3 days after the entry into stationary phase. At this time, the surviving population of bacteria varied between 10^4 and 10^6 CFU ml^{-1}; there was then a more gradual decrease in viability. Colonies obtained from glucose-starved cultures surviving for 8 days were used to inoculate fresh FMC containing 6 mM glucose. The survival of *S. mutans* in these cultures was similar to the original survival pattern (data not shown). This result indicated that the surviving population had not mutated toward long-term survival. The stationary-phase survival of bacteria grown in FMC containing sucrose was similar to that observed for medium containing glucose. Bacteria that were starved for sucrose survived for an average of 11 days in stationary phase, while bacteria in the presence of excess sucrose only survived for an average of 2.5 days (Table 1).

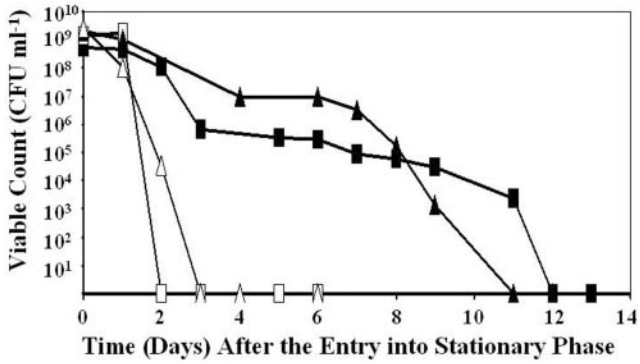

FIG. 1. Survival of *S. mutans* UA159 in batch cultures containing an excess or limiting concentration of glucose. *S. mutans* grown in FMC plus 6 mM glucose (filled rectangles) or THB (filled triangles) was in a glucose-limiting medium, and bacteria grown in FMC plus 100 mM glucose (open rectangles) or THB plus 100 mM glucose (open triangles) were in a medium with excess glucose. Samples were taken after entry into stationary phase, serially diluted, and plated on TH agar. The minimum level of detection for this experiment was 10 CFU ml^{-1}. The results are from one experiment and are representative of three independent experiments.

S. mutans grown in THB (10 mM glucose) reached an OD_{675} of 1.08 at the entry into stationary phase. Bacteria remained viable for an average of 10 days. In stationary phase, the surviving population of bacteria declined to 10^7 CFU ml^{-1} after about 4 days; there was then a gradual decrease in viability (Fig. 1). Bacteria only survived for an average of 2 days in stationary phase if THB was supplemented with 100 mM glucose (Fig. 1).

On day 14, when no culturable bacteria were detected in sugar-limited FMC or THB cultures, an average of 8.3 and 2.9% of the bacteria still had intact membranes as determined by *Bac*Light staining. In cultures with excess sugar, in which culturable bacteria were not detected after 3 days in stationary phase, the percentages were significantly lower, at 0.8% in FMC and 0.2% in THB. *Bac*Light staining was also performed on exponentially growing cells that were treated with 5% formalin or heat killed at 85°C. After the treatments, bacteria could not be cultured on TH agar, yet 0.5% of the formalin-

TABLE 1. Survival of *S. mutans* UA159 in chemically defined medium (FMC) and complex medium (THB) in batch cultures

Culture medium	Survival time (days)[a]	pH[b]	Parameters in presence of lactic acid[c]	
			Days of survival	pH
FMC + 6 mM glucose	10.5 ± 1.9	7.0	1.9 ± 0.2	4.0
FMC + 100 mM glucose	3.0 ± 1.0	4.0	1.9 ± 0.2	4.0
FMC + 3 mM sucrose	11 ± 2.9	7.0	NT	NT
FMC + 100 mM sucrose	2.5 ± 0.7	4.0	NT	NT
THB	11 ± 3.5	5.0	NT	NT
THB + 100 mM glucose	3.5 ± 0.6	4.0	NT	NT

[a] Survival after entry into stationary phase. Data are averages of three independent experiments ± SD.

[b] pH was measured at the time of culture death, which was defined as <10 CFU ml^{-1}.

[c] Lactic acid (80 mM) was added at the entry into stationary phase. Bacteria were allowed to adjust for 1 h and then were plated for surival. The pH was determined immediately after the addition of lactic acid. The results are the averages of three experiments ± SD. NT, not tested for this experiment.

killed bacteria and 1.6% of the heat-killed bacteria stained positive for intact membranes.

The culture pH was measured when bacteria entered stationary phase and again when no bacteria could be recovered from the culture in order to determine if a change in pH may have been responsible for the loss of viable bacteria (Table 1). The pH of FMC at the time of inoculation was 7.0. The glucose-starved cultures maintained a pH of 7.0 even when viable bacteria could no longer be recovered, indicating that the culture did not die because of a fall in pH. However, the pH dropped to 4.0 in cultures containing excess glucose by the time the bacteria entered stationary phase. At this time, the viable count was $>10^8$ CFU ml^{-1}. Bacteria remained viable at a pH of 4.0 for 2 to 4 days after the entry into stationary phase with excess glucose, but they could no longer be cultured after 4 days. To determine if a lower pH could shorten survival from the 11 days obtained for conditions of sugar starvation, we added lactic acid to glucose-starved cultures as the bacteria entered stationary phase, lowering the culture pH to 4.0. After the addition of lactic acid, the cultures were incubated for 1 h at 37°C before the first sample was taken. The initial viable counts did not differ greatly between treated and untreated cultures (2.3×10^9 and 1.8×10^9 CFU ml^{-1}, respectively). Bacteria in glucose-starved cultures in which the pH was reduced to 4.0 survived for only 2 days in stationary phase, similar to the survival of bacteria grown in the presence of excess glucose. Lowering the culture pH to 4.0 with HCl gave similar results (data not shown).

The pHs of sugar-limited THB cultures dropped from 7.0 to 5.0 by the time the bacteria entered stationary phase. The bacteria survived in THB at a pH of 5.0 for an average of 10 days. When THB was supplemented with 100 mM glucose, the culture pH decreased to 4.0 as the bacteria entered stationary phase, and the bacteria survived for only 3.5 days, similar to the results for cultures grown in FMC with excess glucose (Table 1).

Biofilm development in flow chambers. Monospecies biofilms of *S. mutans* UA159 were grown in a flow cell biofilm chamber. After bacteria were inoculated into the chamber, medium was pumped through the chamber at a rate of 200 μl min^{-1}, a rate that is representative of minimum salivary flow (10). Planktonic bacteria were washed out of the chamber so that the biofilm formed from bacteria that had adhered rapidly to the glass coverslip. After inoculation, the biofilms were allowed to grow for up to 24 h in the presence of sucrose or glucose, with chambers removed periodically for imaging. Figure 2 shows the developmental patterns at 2, 7, 14, and 24 h. After 2 h, bacteria were observed adhering to the glass coverslip. After 7 h of growth, two distinct developmental patterns were observed, depending on the carbon source. When they were grown in glucose, the adherent bacteria grew as long chains, allowing the bacteria to spread across the coverslip. The bacteria eventually formed a confluent layer that reached a depth ranging from 12 to 80 μm (Fig. 2, 24 h). When sucrose was used as the sole carbon source, we observed the formation of distinct microcolonies (Fig. 2, 7 h). These microcolonies continued to grow in both diameter and depth over time. The final structures were relatively heterogeneous, reaching approximately 50 to 100 μm in diameter and depths ranging from 30 to >300 μm (Fig. 2, 24 h). We also observed that individual microcolonies could grow into one another, forming larger, clumped structures (Fig. 2, 24 h).

Survival of *S. mutans* UA159 in sugar-starved biofilms. Biofilms were established for 16 to 20 h in FMC containing 3 mM sucrose or 6 mM glucose. Once established, the biofilms were starved by replacing the medium reservoir with fresh FMC lacking sucrose or glucose. Biofilm survival was monitored by culturing dispersed bacteria on TH agar. After the initial 16- to 20-h growth period, a chamber was removed, and the biofilm was scraped from the cover glass and transferred to 1 ml of RTF. Bacteria were plated on TH agar before and after the biofilm bacteria were dispersed by sonication. This procedure was repeated at different intervals following the onset of starvation (Table 2). After the initial 16 to 20 h (day 0) in the presence of sucrose, an average of 2.1×10^8 CFU per chamber were obtained. The number of culturable bacteria decreased as the bacteria were starved for longer periods of time. In contrast to sucrose-starved batch cultures, which still had 2.2×10^6 CFU ml^{-1} after 4 days, bacteria were no longer culturable from biofilms that had been starved of sucrose for 4 days, although the pH of the effluent remained at 7.0. Glucose-starved biofilms behaved similarly to sucrose-starved biofilms and differently from glucose-starved batch cultures (Table 2).

*Bac*Light staining indicated that a significant population of bacteria had intact membranes after 5 days of sucrose starvation in biofilms (Fig. 3), even though there were no culturable bacteria on TH agar. In addition, no bacteria could be recovered on any other medium tested, including FMC agar with 3 mM sucrose (±0.5 M sorbitol as an osmotic stabilizer), dilute TH agar (10 or 50%), or TH agar supplemented with 0.1, 0.5, or 1.0% sodium pyruvate, which has been shown to resuscitate bacteria that are unable to grow on standard media (17, 23, 24, 28, 39). The number of bacteria with intact membranes slowly decreased with time, yet a large population of bacteria still stained positive for intact membranes after 12 days of sucrose starvation. Treatment with 10% formalin was not sufficient to disrupt the membranes (data not shown) of bacteria within a growing biofilm, as a significant number of bacteria still had

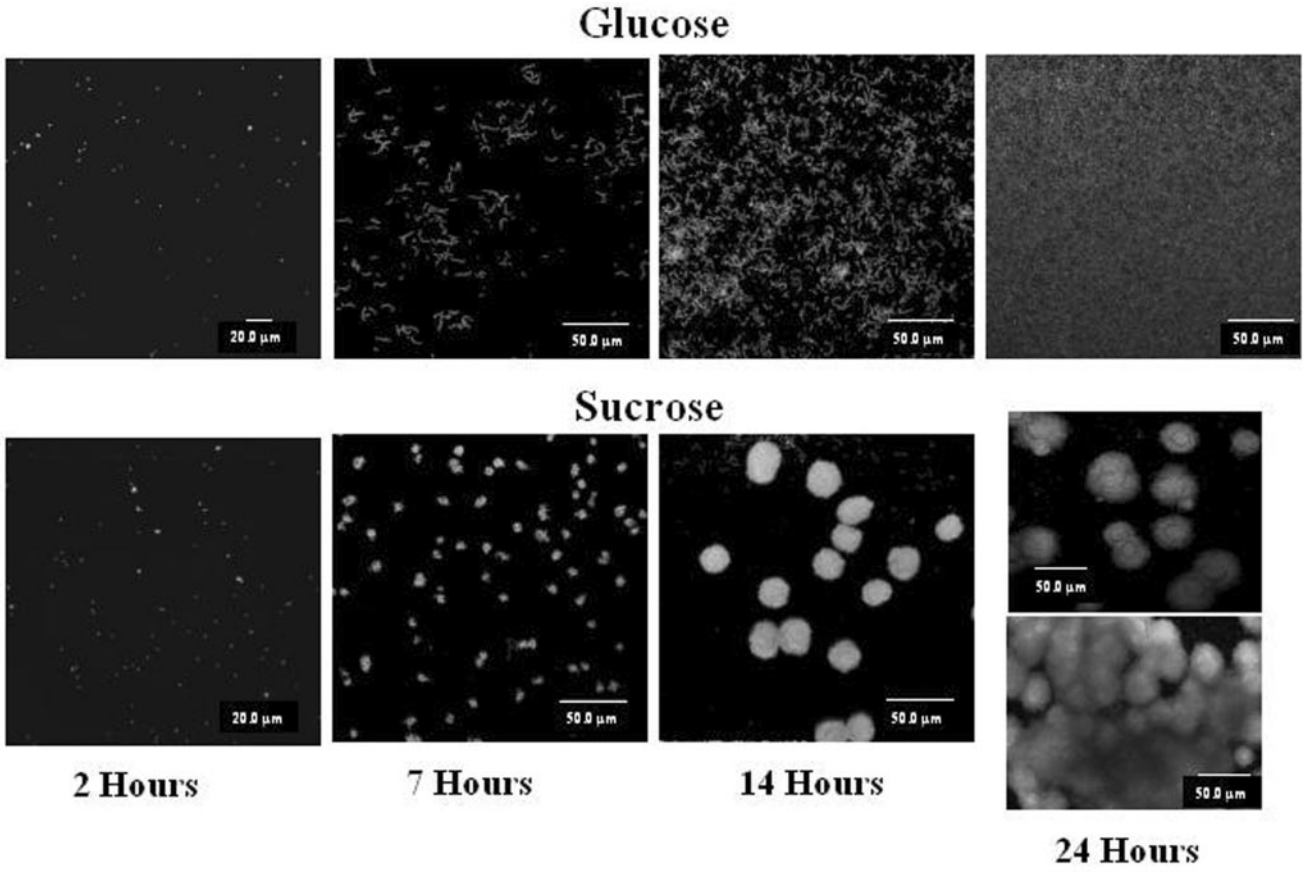

FIG. 2. *S. mutans* UA159 biofilm development in the presence of glucose or sucrose. Flow chambers were used to grow biofilms as previously described (27). Flow chambers were maintained in parallel, with chambers removed for imaging after 2, 7, 14, and 24 h of growth in FMC containing glucose or sucrose. The biofilms were stained with the *Bac*Light viability stain and were imaged by confocal scanning laser microscopy. Stack images were created from individual slices from both the SYTO 9 and PI channels by use of the Olympus Fluoview system. Images were converted to a gray scale in Adobe Photoshop 7.0. Mature biofilms in the presence of sucrose were observed to contain both isolated (24 h, top) and clumped (24 h, bottom) microcolonies. Bar, 20 μm for the 2-h images and 50 μm for the 7- to 24-h images. The images are representative of two independent experiments.

intact membranes even though they could not be cultured on TH agar. The membranes of bacteria growing within a biofilm were disrupted by heating the biofilm at 85°C for 1 h (data not shown).

Mucin extends survival in both sugar-starved batch cultures and biofilms. During periods of dietary sugar starvation, specific environmental factors may contribute to the survival of *S. mutans*. One potential factor is mucin, the major glycoprotein found in saliva (38). In order to test this possibility, we grew batch cultures in FMC containing 6 mM glucose, with and without 0.5% mucin. *S. mutans* was observed to have an increased growth rate when grown in the presence of mucin (Fig.

TABLE 2. Survival of *S. mutans* in sugar-starved biofilms and batch cultures

Day	Growth in biofilms[a] (CFU per chamber [experiment 1/experiment 2])		Growth in batch cultures[b] (CFU ml^{-1} [experiment 1/experiment 2])	
	Sucrose	Glucose	Sucrose	Glucose
0	$4.0 \times 10^8/1.5 \times 10^7$	$4.0 \times 10^7/5.5 \times 10^7$	$4.7 \times 10^9/5.5 \times 10^8$	$1.4 \times 10^9/6.0 \times 10^8$
1	$3.5 \times 10^6/7.0 \times 10^5$	$2.0 \times 10^6/5.5 \times 10^5$	$3.6 \times 10^7/2.5 \times 10^8$	$4.5 \times 10^8/6.6 \times 10^8$
2	$4.5 \times 10^4/2.0 \times 10^4$	$1.8 \times 10^3/7.0 \times 10^3$	$2.0 \times 10^7/4.2 \times 10^7$	$1.5 \times 10^8/1.9 \times 10^8$
3	$1.7 \times 10^2/2.0 \times 10^2$	$5.5 \times 10^2/9.0 \times 10^1$	$1.3 \times 10^7/3.0 \times 10^6$	$9.5 \times 10^5/2.7 \times 10^7$
4	ND/ND	ND/ND	$3.0 \times 10^6/2.0 \times 10^6$	$1.5 \times 10^5/8.5 \times 10^5$

[a] Biofilms were established in 3 mM sucrose or 6 mM glucose. The medium in the reservoir was replaced with FMC lacking glucose or sucrose (starvation medium) after 17 or 18 h of growth (day 0). Survival was measured by the culturing of dispersed biofilms on TH agar. ND, not detected, with the minimum level of detection being <10 CFU per chamber.

[b] *S. mutans* was grown in FMC containing 3 mM sucrose or 6 mM glucose, and survival was measured by culturing the bacteria on TH agar. The data shown here are from two independent experiments.

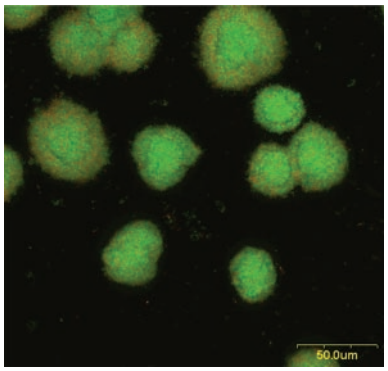

FIG. 3. *Bac*Light staining of *S. mutans* UA159 in a biofilm after 5 days of sucrose starvation. The biofilm was established for 16 h in FMC with 3 mM sucrose, after which the medium reservoir was replaced with biofilm starvation medium. The biofilm was maintained in the starvation medium for 5 days and then stained with *Bac*Light and imaged by confocal scanning laser microscopy. Individual sections were collected and compiled to create a maximum projection image by use of the Olympus Fluoview system. The image is representative of three independent experiments.

4), although the bacteria entered stationary phase at approximately the same optical density as bacteria grown in the absence of mucin (Fig. 4B). The presence of mucin in addition to sugar increased the final mass yield of the cultures at the entry into stationary phase (Fig. 4A), even though it was previously reported that *S. mutans* could not grow with mucin as its sole carbon source (38). The addition of mucin to the growth medium extended the stationary-phase survival of glucose-starved batch cultures. Bacteria that were starved for glucose survived for an average of 11 (±2) days, but in the presence of 0.5% mucin the average time of survival increased to 26 (±8) days. Similar results were observed when FMC was supplemented with 1 or 2% mucin, with average survival times of 26 (±6) and 22 (±9) days, respectively. The addition of 0.5% mucin to batch cultures grown in FMC with 100 mM glucose did not enhance survival; the pH of these cultures dropped to 4.0 when the bacteria entered stationary phase, and the bacteria survived for only 2 days.

Mucin was also tested for its ability to enhance the survival of *S. mutans* in glucose- and sucrose-starved biofilms. Biofilms were grown in the presence of 3 mM sucrose and 0.5% mucin. The addition of mucin to the growth medium did not appear to affect biofilm development (data not shown). The subsequent starvation medium also contained 0.5% mucin. After 4 days of sucrose starvation, biofilms in the presence of mucin had an average of 1.8×10^6 CFU per chamber; in contrast, biofilms that were starved in the absence of mucin contained no culturable bacteria (Table 3). Biofilms that were starved for sucrose in the presence of mucin were monitored for 12 days, at which time they still contained 2.7×10^5 CFU per chamber. The survival of bacteria from glucose-starved biofilms was also extended by the addition of 0.5% mucin. Glucose-starved biofilms in the presence of 0.5% mucin were monitored for 8 days, at which time they still contained an average of 5.3×10^5 CFU per chamber.

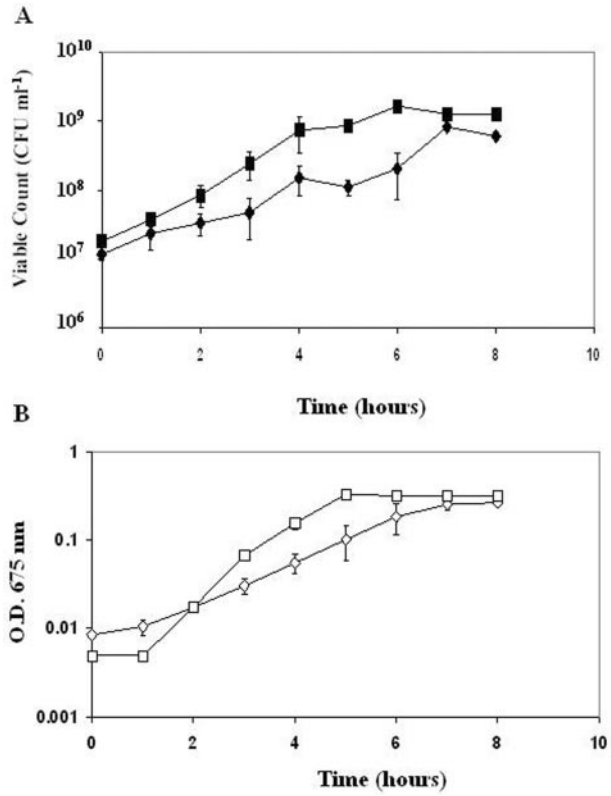

FIG. 4. Growth of *S. mutans* UA159 in FMC containing 6 mM glucose and 0.5% mucin in batch cultures. Growth curves are shown for *S. mutans* UA159 in FMC plus 6 mM glucose (diamonds), FMC plus 6 mM glucose and 0.5% mucin (squares). An overnight culture grown in FMC with 24 mM glucose was diluted 25-fold into fresh medium, and growth was assayed by measuring the CFU on TH agar (A) or OD_{675} (B). The growth curves measured by optical densities and CFU were from independent experiments. The data shown are the averages of three experiments ± standard deviations.

DISCUSSION

S. mutans is a member of the normal oral flora residing in dental plaque biofilms. The fate of the bacteria within a mature biofilm once one has been established on tooth enamel remains relatively uncharacterized. We wanted to identify conditions that would allow for the persistence of *S. mutans*. To do this, we compared the survival of *S. mutans* in glucose- and sucrose-starved batch cultures and biofilms. Sugar starvation

TABLE 3. Effects of mucin on survival of *S. mutans* in sucrose-starved biofilms[a]

Day	CFU per chamber (experiment 1/experiment 2)	
	Sucrose	Sucrose + mucin
0	$4.0 \times 10^8/1.5 \times 10^7$	$2.5 \times 10^6/1.3 \times 10^7$
4	ND/ND	$1.5 \times 10^6/3.5 \times 10^6$
8	ND/ND	$2.5 \times 10^5/1.6 \times 10^4$
12	ND/ND	$3.5 \times 10^4/5.0 \times 10^5$

[a] Biofilms were established in the presence of 3 mM sucrose or 3 mM sucrose plus 0.5% mucin. The medium in the reservoir was replaced with FMC lacking sucrose (starvation medium) after 17 or 18 h of growth (day 0). The starvation medium for bacteria grown in the presence of mucin also contained mucin. ND, not detected, with the minimum level of detection being <10 CFU per chamber.

increased the survival time in batch cultures to 11 days, from 3 days when sugar was present in excess; survival depended at least in part on the culture pH remaining neutral in the sugar-limited cultures. The surviving population did not undergo a genetic change toward long-term survival. When colonies derived from a culture that had survived for 8 days with glucose starvation were used as an inoculum, identical survival patterns were observed for them as for the original culture.

Sugar starvation in biofilms did not result in comparable survival to that of batch cultures. Bacteria in biofilms remained culturable for only 3 days after the imposition of sugar starvation, although the pH remained at 7.0. There are a number of possible factors that could account for the differences in survival observed between biofilm and batch culture bacteria. First, the environments of the bacteria differed between the two systems. The biofilms were formed in a flow cell chamber, and medium was continuously pumped through the chamber at a rate of 200 μl min^{-1}. In this system, planktonic bacteria were continuously washed out of the chamber. Since fresh medium was constantly pumped through the chamber, starvation was brought about by changing the medium reservoir to fresh FMC lacking the sugar used to establish the biofilm instead of by allowing the bacteria to gradually deplete the medium of sugar, as in batch cultures. Differences in survival have been reported for *Rhizobium leguminosarum* depending on whether sugar starvation was abrupt or gradual (35). In addition, stationary-phase bacteria in a batch culture were present in a spent medium, whereas stationary-phase biofilm bacteria were continuously washed with fresh FMC. The spent medium may provide a better environment for survival. It is possible that another nutrient besides the carbon source is depleted and that its depletion contributes to survival. Fresh FMC would also lack any molecules secreted by the bacteria during exponential growth or at the entry into stationary phase that may be essential for the uptake of nutrients needed for long-term persistence (e.g., siderophores). Another possibility is that quorum sensing may contribute to survival; a secreted signal could accumulate in the spent medium but not in the flow cell chambers.

A second factor, apart from differences in the medium, is the physiological difference of *S. mutans* grown in biofilms from that grown in batch cultures. *S. mutans* organisms grown in biofilms have been shown to be repressed in terms of respiration (26) and are approximately 600 times more competent than planktonic bacteria (15). Differential gene expression has also been shown for *S. mutans* grown in biofilms compared to planktonic bacteria (3, 44). Thus, biofilm bacteria may respond differently than planktonic bacteria to environmental stresses, such as sugar starvation, that affect stationary-phase survival.

The structures of biofilms grown in the presence of glucose or sucrose differed. *S. mutans* grows at similar exponential rates in both sugars, but with sucrose the bacteria form large aggregates because of the production of extracellular glucans (5). These glucans are important for the development of a mature biofilm architecture (1, 20, 37). However, in the absence of sucrose, *S. mutans* is still able to colonize surfaces through interactions with its major protein adhesion P1 (11, 12). This behavior may explain the confluent layer of bacteria attached to coverslips that we observed when glucose was provided as the sole carbon source. The distinct microcolonies observed in the biofilms when sucrose was provided as the sole carbon source were presumably dependent on the production of extracellular glucans that allowed the bacteria to adhere to one another as well as to the glass coverslip. Our results showed that the survival times of glucose- and sucrose-starved *S. mutans* in biofilms were similar despite the different biofilm structures, indicating that survival was not dependent on the architecture, and in particular, not on bacterial aggregation.

The survival time of *S. mutans* was short compared to that of other streptococcal species (6, 9, 30, 36; D. N. Wood and B. A. Buttaro, submitted for publication) that can persist for months to years, and in contrast to what is observed for other bacteria, a rich medium (THB) did not increase survival times (9; Wood and Buttaro, submitted). It is possible that long-term persistence requires additional factors that are not present in FMC or THB. Mucin is a major glycoprotein that is found in saliva (38). Pig gastric mucin was successfully used as a substrate for the growth of a complex oral microflora, but it could not be used as a carbon and energy source for the growth of mutans streptococci in a chemically defined medium (38). However, mucin appeared to affect the metabolic activity of *S. mutans* grown in FMC plus 6 mM glucose, as the bacterial growth rate increased, allowing the bacteria to enter stationary phase sooner than bacteria grown in the absence of mucin (Fig. 4). Mucin increased the final mass yield in the cultures, indicating that it may serve as a carbon source.

Importantly, bacteria were observed to persist longer in the presence of mucin in both biofilms and batch cultures. However, the role of mucin remains to be defined. It is possible that after the end of exponential growth, *S. mutans* can grow slowly on mucin as a secondary carbon source after priming with another carbon source, such as glucose or sucrose, and that this slow growth allows the bacteria to persist longer in both batch cultures and biofilms. It is also possible that mucin binds to the bacteria, coating them and either protecting them or signaling them to survive. The survival with mucin was still relatively short (~1 month), suggesting that other factors necessary for survival were still missing. Alternatively, it may be that because of its natural environment, *S. mutans* is not adapted for long-term survival.

Finally, *Bac*Light staining for viability did not correlate with culturability under a range of conditions. *Bac*Light stains for the presence of bacteria with intact membranes, and these are inferred to be viable. However, depending on the condition leading to cell death (e.g., starvation versus formalin killing or heat killing), we saw various numbers of bacteria with intact membranes within the biofilms. In particular, many bacteria with intact membranes were observed in biofilms after a treatment with 10% formalin for 1 h. We cannot rule out the possibility that the bacteria with intact membranes are viable but not culturable in rich media, poor media, or redox-protected media. However, we concluded that the presence of bacteria with intact membranes is not a reliable indicator of the viability of *S. mutans* within biofilms.

Taken together, these results indicate that the presence of mucin prolongs the survival of *S. mutans* in sugar-starved biofilms. Further characterization of the effect of mucin on *S. mutans* persistence may lead to a better understanding of the biological state of the bacteria in a mature biofilm.

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REFERENCES

1. **Banas, J. A., and M. M. Vickerman.** 2003. Glucan-binding proteins of the oral streptococci. Crit. Rev. Oral Biol. Med. **14:**89–99.
2. **Burne, R. A.** 1998. Oral streptococci. Products of their environment. J. Dent. Res. **77:**445–452.
3. **Burne, R. A., Y. Y. Chen, and J. E. Penders.** 1997. Analysis of gene expression in *Streptococcus mutans* in biofilms in vitro. Adv. Dent. Res. **11:**100–109.
4. **Clements, M. O., S. P. Watson, R. K. Poole, and S. J. Foster.** 1999. CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. J. Bacteriol. **181:**501–507.
5. **Daneo-Moore, L., B. Terleckyj, and G. D. Shockman.** 1975. Analysis of growth rate in sucrose-supplemented cultures of *Streptococcus mutans*. Infect. Immun. **12:**1195–1205.
6. **Giard, J. C., A. Hartke, S. Flahaut, P. Boutibonnes, and Y. Auffray.** 1997. Glucose starvation response in *Enterococcus faecalis* JH2–2: survival and protein analysis. Res. Microbiol. **148:**27–35.
7. **Hazlett, K. R., J. E. Mazurkiewicz, and J. A. Banas.** 1999. Inactivation of the *gbpA* gene of *Streptococcus mutans* alters structural and functional aspects of plaque biofilm which are compensated by recombination of the *gtfB* and *gtfC* genes. Infect. Immun. **67:**3909–3914.
8. **Idone, V., S. Brendtro, R. Gillespie, S. Kocaj, E. Peterson, M. Rendi, W. Warren, S. Michalek, K. Krastel, D. Cvitkovitch, and G. Spatafora.** 2003. Effect of an orphan response regulator on *Streptococcus mutans* sucrose-dependent adherence and cariogenesis. Infect. Immun. **71:**4351–4360.
9. **Kim, W. S., J. H. Park, J. Ren, P. Su, and N. W. Dunn.** 2001. Survival response and rearrangement of plasmid DNA of *Lactococcus lactis* during long-term starvation. Appl. Environ. Microbiol. **67:**4594–4602.
10. **Koshimune, S., S. Awano, K. Gohara, E. Kurihara, T. Ansai, and T. Takehara.** 2003. Low salivary flow and volatile sulfur compounds in mouth air. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **96:**38–41.
11. **Lee, S. F., A. Progulske-Fox, G. W. Erdos, D. A. Piacentini, G. Y. Ayakawa, P. J. Crowley, and A. S. Bleiweis.** 1989. Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/II). Infect. Immun. **57:**3306–3313.
12. **Lee, S. F., and T. L. Boran.** 2003. Roles of sortase in surface expression of the major protein adhesion P1, saliva-induced aggregation and adherence, and cariogenicity of *Streptococcus mutans*. Infect. Immun. **71:**676–681.
13. **Lemos, J. A., T. A. Brown, Jr., and R. A. Burne.** 2004. Effects of RelA on key virulence properties of planktonic and biofilm populations of *Streptococcus mutans*. Infect. Immun. **72:**1413–1440.
14. **Li, Y. H., M. N. Hanna, G. Svensater, R. P. Ellen, and D. G. Cvitkovitch.** 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. J. Bacteriol. **183:**6875–6884.
15. **Li, Y. H., P. C. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch.** 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J. Bacteriol. **183:**897–908.
16. **Li, Y. H., N. Tang, M. B. Aspiras, P. C. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch.** 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J. Bacteriol. **184:**2699–2708.
17. **Lleo, M. M., B. Bonato, M. C. Tafi, C. Signoretto, M. Boaretti, and P. Canepari.** 2001. Resuscitation rate in different enterococcal species in the viable but non-culturable state. J. Appl. Microbiol. **91:**1095–1102.
18. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
19. **Loesche, W. J., R. N. Hockett, and S. A. Syed.** 1972. The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch. Oral Biol. **17:**1311–1325.
20. **Matsumura, M., T. Izumi, M. Matsumoto, M. Tsuji, T. Fujiwara, and T. Ooshima.** 2003. The role of glucan-binding proteins in the cariogenicity of *Streptococcus mutans*. Microbiol. Immunol. **47:**213–215.
21. **McNeill, K., and I. R. Hamilton.** 2003. Acid tolerance response of biofilm cells of *Streptococcus mutans*. FEMS Microbiol. Lett. **221:**25–30.
22. **Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi.** 2003. Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. Infect. Immun. **71:**1972–1979.
23. **Mizunoe, Y., S. N. Wai, T. Ishikawa, A. Takade, and S. Yoshida.** 2000. Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. FEMS Microbiol. Lett. **186:**115–120.
24. **Mizunoe, Y., S. N. Wai, A. Takade, and S. Yoshida.** 1999. Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H_2O_2-degrading compounds. Arch. Microbiol. **172:**63–67.
25. **Munro, C., S. M. Michalek, and F. L. Macrina.** 1991. Cariogenicity of *Streptococcus mutans* V403 glucosyltransferase and fructosyltransferase mutants constructed by allelic exchange. Infect. Immun. **59:**2316–2323.
26. **Nguyen, P. T., J. Abranches, T. N. Phan, and R. E. Marquis.** 2002. Repressed respiration of oral streptococci grown in biofilms. Curr. Microbiol. **44:**262–266.
27. **Palmer, R. J., Jr.** 1999. Microscopy flowcells: perfusion chambers for real-time study of biofilms. Methods Enzymol. **310:**160–166.
28. **Reissbrodt, R., I. Rienaecker, J. M. Romanova, P. P. Freestone, R. D. Haigh, M. Lyte, H. Tschape, and P. H. Williams.** 2002. Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* from the viable but nonculturable state by heat-stable enterobacterial autoinducer. Appl. Environ. Microbiol. **68:**4788–4794.
29. **Smeulders, M. J., J. Keer, R. A. Speight, and H. D. Williams.** 1999. Adaptation of *Mycobacterium smegmatis* to stationary phase. J. Bacteriol. **181:**270–283.
30. **Stuart, M. R., L. S. Chou, and B. C. Weimer.** 1999. Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **65:**665–673.
31. **Svensater, G., O. Bjornsson, and I. R. Hamilton.** 2001. Effect of carbon starvation and proteolytic activity on stationary-phase acid tolerance of *Streptococcus mutans*. Microbiology **147:**2971–2979.
32. **Svensater, G., U. B. Larsson, E. C. Greif, D. G. Cvitkovitch, and I. R. Hamilton.** 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol. Immunol. **12:**266–273.
33. **Svensater, G., B. Sjogreen, and I. R. Hamilton.** 2000. Multiple stress responses in *Streptococcus mutans* and the induction of general and stress-specific proteins. Microbiology **146:**107–117.
34. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11:**649–655.
35. **Thorne, S. H., and H. D. Williams.** 1997. Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. *phaseoli*: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. **179:**6894–6901.
36. **Trainor, V. C., R. K. Udy, P. J. Bremer, and G. M. Cook.** 1999. Survival of *Streptococcus pyogenes* under stress and starvation. FEMS Microbiol. Lett. **176:**421–428.
37. **Tsumori, H., and H. Kuramitsu.** 1997. The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. Oral Microbiol. Immunol. **12:**274–280.
38. **van der Hoeven, J. S., C. W. van den Kieboom, and P. J. Camp.** 1990. Utilization of mucin by oral Streptococcus species. Antonie Leeuwenhoek **57:**165–172.
39. **Wai, S. N., Y. Mizunoe, A. Takade, and S. Yoshida.** 2000. A comparison of solid and liquid media for resuscitation of starvation- and low-temperature-induced nonculturable cells of *Aeromonas hydrophila*. Arch. Microbiol. **173:**307–310.
40. **Watson, S. P., M. O. Clements, and S. J. Foster.** 1998. Characterization of the starvation-survival response of *Staphylococcus aureus*. J. Bacteriol. **180:**1750–1758.
41. **Wen, Z. T., and R. A. Burne.** 2002. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. Appl. Environ. Microbiol. **68:**1196–1203.
42. **Yamashita, Y., W. H. Bowen, R. A. Burne, and H. K. Kuramitsu.** 1993. Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. Infect. Immun. **61:**3811–3817.
43. **Yoshida, A., and H. K. Kuramitsu.** 2002. Multiple *Streptococcus mutans* genes are involved in biofilm formation. Appl. Environ. Microbiol. **68:**6283–6291.
44. **Yoshida, A., and H. K. Kuramitsu.** 2002. *Streptococcus mutans* biofilm formation: utilization of a *gtfB* promoter-green fluorescent protein (P*gtfB*::*gfp*) construct to monitor development. Microbiology **148:**3385–3394.
45. **Zhu, M., S. Takenaka, M. Sato, and E. Hoshino.** 2001. Influence of starvation and biofilm formation on acid resistance of *Streptococcus mutans*. Oral Microbiol. Immunol. **16:**24–27.