Regulation of the *gtfBC* and *ftf* genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate

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***Streptococcus mutans* produces a number of extracellular sucrose-metabolizing enzymes that contribute to the ability of the organism to cause dental caries, including three glucosyltransferases, the products of the *gtfB*, *gtfC* and *gtfD* genes, and a fructosyltransferase, encoded by the *ftf* gene. To better understand the regulation of the expression of these genes under environmental conditions that more closely mimic those in dental plaque, two strains of *S. mutans* harbouring fusions of the *gtfBC* (SMS102) and *ftf* (SMS101) promoters to a chloramphenicol acetyltransferase (CAT) gene were examined in biofilms formed *in vitro*. The strains were grown in a Rotatorque biofilm reactor in a tryptone-yeast extract-sucrose medium. CAT specific activity in biofilm cells was measured at quasi-steady state or following additions of 25 mM sucrose or glucose, with or without pH control. After approximately 10 generations of biofilm growth, the *ftf* and *gtfBC* genes of *S. mutans* were found to be expressed at levels different from those reported for planktonic cells growing under otherwise similar conditions. The expression of these genes was induced by the addition of sucrose to the quasi-steady-state cultures. Expression of the *gtfBC* genes was influenced by environmental pH, since CAT specific activities in quasi-steady-state biofilms of strain SMS102 grown without pH control were twice those produced by cells grown with pH control. Moreover, addition of glucose to quasi-steady-state biofilms resulted in increased expression of the *gtfBC*-cat fusion, although the magnitude of the induction was less than that seen with sucrose. The effect of pH on *ftf* expression was negligible. A modest and transient induction of *ftf* was observed in biofilms pulsed with excess glucose and the kinetics and level of induction of *ftf* by excess carbohydrate were dependent on the pH of the biofilms. This study demonstrates that the type and amount of carbohydrate and the environmental pH have a major influence on transcription of the *gtfBC* and *ftf* genes when the organisms are growing in biofilms, and provides evidence for previously undisclosed regulatory circuits for exopolysaccharide gene expression in *S. mutans*.**

Keywords: exopolysaccharides, pathogenesis, glucans, dental caries, dental plaque

INTRODUCTION

Streptococcus mutans has been described as an 'obligate biofilm' organism (Burne, 1998b), and is considered to

be a principal aetiological agent of dental caries (Hamada & Slade, 1980). The ability of *S. mutans* to initiate caries depends on several virulence-associated traits, including (i) initial adherence to the tooth through high-affinity adhesins, such as the SpaP protein, (ii) the ability to synthesize insoluble, extracellular polysaccharides that enhance accumulation and persistence of the organism on the tooth surfaces, (iii) a high capacity to catabolize carbohydrates and produce acids and (iv) the ability to grow and continue to metabolize

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Abbreviation: CAT, chloramphenicol acetyltransferase.

carbohydrates at low pH (Burne, 1998a; Kuramitsu, 1993). Among these virulence attributes, the ability of *S. mutans* to produce extracellular polysaccharides from dietary carbohydrates has been demonstrated to significantly enhance its cariogenicity (Yamashita *et al.*, 1993).

S. mutans synthesizes glucan polymers from sucrose via the actions of three secreted glucosyltransferases (GTFs), encoded by the *gtfB*, *gtfC* and *gtfD* genes (Kuramitsu, 1993). The *gtfB* and *gtfC* genes are in an operon-like arrangement and encode enzymes that produce mainly water-insoluble glucans, whereas the *gtfD* gene, which is not linked to the *gtfBC* locus, encodes an enzyme that catalyses the formation of a water-soluble glucan. It is the water-insoluble glucans made by GtfBC that play important roles in adhesion and accumulation of the organisms on the tooth surfaces, and in establishing the extracellular polysaccharide matrix that is responsible for the structural integrity of dental biofilms. These polysaccharides are also thought to provide the organisms with a unique microenvironment for their growth, metabolism and survival (Bowden & Hamilton, 1998; Liljemark & Bloomquist, 1996; Nakano & Kuramitsu, 1992; Yamashita *et al.*, 1993). *S. mutans* produces a single fructosyltransferase (FTF), the product of the *ftf* gene, which catalyses the synthesis of high-molecular-mass fructans from sucrose (Ebisu *et al.*, 1975; Shiroza & Kuramitsu, 1988). Fructans produced by *S. mutans* are believed to function primarily as extracellular storage compounds that can be metabolized during periods of nutrient deprivation (Burne *et al.*, 1996).

A number of studies indicate that the expression of the genes for the exopolysaccharide-synthesizing enzymes of *S. mutans* is dependent on environmental conditions, including growth rate, pH, carbon source, and whether the organisms are attached to the surfaces (Burne *et al.*, 1997; Hudson & Curtiss, 1990; Kiska & Macrina, 1994; Wexler *et al.*, 1993). Also, the use of *gtf* and *ftf* gene fusion strains in a continuous-flow biofilm fermenter has shown that organisms growing in thicker, mature (7-d-old) biofilms have higher levels of expression of the *gtfBC* genes compared with cells grown in suspension or in thinner (2-d-old) biofilms (Burne *et al.*, 1997; Hudson & Curtiss, 1990; Kiska & Macrina, 1994; Wexler *et al.*, 1993). In contrast, *ftf* was found to be dramatically down-regulated in 7 d biofilms compared with 2 d biofilms. The specific mechanisms governing regulation of exopolysaccharide synthesis in *S. mutans* biofilms have yet to be discovered. In this study, we test the hypothesis that pH or changes in carbohydrate source and concentration in biofilms influence expression of *gtf* and *ftf* in mature biofilms.

METHODS

Bacterial strains, growth media and biofilm reactor conditions. The bacterial strains utilized in this study were *S. mutans* harbouring *ftf-cat* (SMS101) or *gtfBC-cat* (SMS102) gene fusions. The construction of these strains and their genotypic stabilities are detailed elsewhere (Burne *et al.*,

1997; Hudson & Curtiss, 1990; Wexler *et al.*, 1993). The stability of the gene fusions in biofilms was again confirmed in this study by quantifying biofilm bacteria on selective and non-selective media, as previously detailed (Burne *et al.*, 1997; Li *et al.*, 2000). The strains were routinely maintained on brain heart infusion (BHI) agar (Difco) supplemented with 10 µg erythromycin ml⁻¹. For the development of biofilms, a tryptone-yeast extract base medium (TY; Burne *et al.*, 1987) was diluted twofold and supplemented with 10 mM sucrose and 10 µg erythromycin ml⁻¹, or with glucose (5 mM) and 10 µg erythromycin ml⁻¹. In addition, the medium contained 50 mM potassium phosphate buffer (pH 7.8) for the experiments requiring pH control, or 90 mM KCl for those not requiring pH control, to ensure that the potassium ion concentration was similar in all cases. Mono-species biofilms were developed in a modified Rotatorque biofilm reactor (Burne *et al.*, 1997) with a working volume of 0.6 l. The Rotatorque is a stirred tank biofilm reactor that can be run in either batch or continuous mode. For this study, continuous culture was used by pumping medium at a fixed dilution rate and the biofilms were formed on slides that were inserted on the inner wall of the vessel. As configured for this study (Li *et al.*, 2000), the Rotatorque reactor allowed biofilm formation on 12 polystyrene slides, each with a mean exposed surface area of 31 cm² for biofilm accumulation. After inoculation with 10 ml of an overnight culture of *S. mutans* SMS101 or SMS102, medium was pumped into the vessel at a constant rate of 60 ml h⁻¹ ('dilution rate', $D = 0.1 \text{ h}^{-1}$). Samples were taken of quasi-steady-state biofilms and of biofilms pulsed with glucose or sucrose. Since bacteria in a biofilm are physiologically heterogeneous, a true steady state is not achieved, so the term quasi-steady state is used here instead. Cultures were designated to have reached quasi-steady state after the equivalent of 10 mean generation times using calculations detailed elsewhere (Burne & Chen, 1998). The speed of the rotating inner drum of the Rotatorque was kept constant at 75 r.p.m. and the temperature was maintained by immersion of the vessel in a 37 °C circulating water bath.

Assessment of physical characteristics of *S. mutans* biofilms.

Biofilm dry weights were determined by mechanically dissociating the biofilms from the slides, collecting the material by centrifugation, washing once with dH₂O, lyophilizing the sample and weighing. For measurement of total carbohydrate in biofilms, the lyophilized samples were resuspended in 5 ml dH₂O and the anthrone method (Dubois *et al.*, 1956) was used to measure total carbohydrate using glucose as the standard. Bacterial viability in biofilms was estimated by direct microscopic enumeration and plate counts with cells prepared as follows. Biofilms were collected, washed once, and resuspended in 5 ml of reduced transport fluid (Loesche & Syed, 1973) at pH 7.2. The samples were then subjected to a gentle sonication at a setting of 150 W for 20 s to break bacterial chains, and the samples were split into two parts. The cell suspensions for viable cell counts were decimally diluted and directly inoculated by a spiral plating system (Autoplate model 3000; Spiral Biotech) onto BHI agar supplemented with 10 µg erythromycin ml⁻¹. The viable counts were made after the plates were cultivated at 37 °C for 24 h in a 5% CO₂ aerobic atmosphere. Microscopic enumeration of bacteria was conducted as described by Koch (1994) using a Petroff-Hausser counting chamber. The ratio of viable cell counts to the counts obtained by visual enumeration was expressed as percentage viable cells. The formation and spatial distribution of biofilms on the surfaces were also assessed by using phase-contrast microscopy, as previously described (Burne *et al.*, 1997; Li *et al.*, 2000).

In situ measurement of biofilm pH. The pH of the biofilms was measured as previously detailed (Li *et al.*, 2000) using a superminiature, Beetrode pH electrode (model MEPH3L; World Precision Instruments). Briefly, after the cultures reached quasi-steady state (Burne & Chen, 1998), slides with biofilms were removed from the vessel and placed on end on a paper towel to allow excess medium to be absorbed from the end of the slides. The micro-reference electrode, which was connected through the Bee-Cal adapter and two cables to the pH probe and a standard pH meter, was positioned in the biofilm to be partially immersed in the biomass. *In situ* measurement of pH was conducted immediately by placing the tip of the pH probe into biofilms and a series of pH readings was recorded from a minimum of 30 different sites selected at random.

Carbohydrate pulsing and biofilm sampling. Quasi-steady-state biofilms, or biofilms which had been pulsed with either glucose or sucrose (25 mM) immediately after initial biofilm sampling (T_0), were used for measurements of chloramphenicol acetyltransferase (CAT) activity expressed from the *gtf* or *ftf* promoters. Subsequently, the biofilms were sampled by removing three slides at 15, 30 and 60 min after the carbohydrate pulse. The biofilms were mechanically dissociated from the slides by scraping with a sterile razor blade into 40 ml ice-cold Tris/HCl (10 mM, pH 7.8) and were centrifuged at 8000 *g* for 10 min at 4 °C. The cell pellets were washed twice and resuspended in 1 ml of the same buffer for the preparation of cell-free lysates. The pH profiles in the liquid phase at each sampling time were recorded by measuring pH in 10 ml of culture fluid.

Preparation of cell-free lysates and CAT assays. Cell-free lysates were prepared for the analysis of CAT activity as previously described (Chen *et al.*, 1998). Briefly, the cell pellets were resuspended in 1 ml Tris buffer in a 2 ml, screw-cap microcentrifuge tube (Sarstedt). One-third volume of pre-chilled (−20 °C) glass beads (0.2 mm mean diameter) was added to the sample, and the cells were homogenized using a Bead Beater (Biospec Products) in four 30 s intervals, with cooling on ice in the intervals. The lysates were centrifuged at 12000 *g* at 4 °C for 15 min and the supernatant material was collected for CAT assays. Protein concentrations of the cell-free lysates were determined by the method of Bradford (1976) using a commercially available reagent (Bio-Rad). CAT activity was measured by a spectrophotometric method (Shaw, 1979) with use of the colorimetric substrate 5,5'-dithio-bis-nitrobenzoic acid (DTNB; Boehringer Mannheim). All assays were performed in triplicate with internal standards containing all reagents except chloramphenicol to account for chloramphenicol-independent reduction of DTNB. One unit of CAT activity was defined as the amount of enzyme needed to catalyse the acetylation of 1 μ mol chloramphenicol min^{-1} (mg protein) $^{-1}$.

RESULTS

Physical characteristics of *S. mutans* biofilms at quasi-steady state

The biofilms at quasi-steady state were indistinguishable from biofilms of *S. mutans* and *Streptococcus salivarius* described previously (Burne *et al.*, 1997; Li *et al.*, 2000) (data not shown) and consisted of many microcolonies, aggregates and mats, with a considerable amount of extracellular polymer. As was observed with *S. salivarius* biofilm formation (Li *et al.*, 2000), biofilms of

S. mutans formed more efficiently at low pH and the proportion of cells in the planktonic phase was far lower in cultures grown at lower pH values. In the cultures with buffered medium (50 mM potassium phosphate buffer, pH 7.8), the dry weight of the biofilms was 180 $\mu\text{g cm}^{-2}$ (Table 1). When the medium was not buffered, the biomass of the biofilms was 50% greater than that of the biofilms formed in buffered medium. Analysis of total carbohydrates showed that the biofilms formed without pH control contained a higher percentage (68%) of carbohydrates than those biofilms formed with pH control (49%), and the total number of cells in the biofilms grown with pH control was about half that of biofilms formed without pH control. *S. mutans* SMS102 showed no significant differences from strain SMS101 in its growth characteristics, final yields or appearance in the biofilm reactor when grown under the same culture conditions (data not shown).

Similar to Rotatorque biofilms of *S. salivarius* (Li *et al.*, 2000), the *S. mutans* biofilms were heterogeneous and achieved thicknesses ranging from 10 to over 300 μm , and significant differences were noted between the pH values measured in the fluid phase and in the biofilms (Table 2). When pH control was imposed by buffering of the medium, the pH of the planktonic phase was near neutrality (pH 6.68 ± 0.15), because the production of acid by the organisms always accompanies growth, and the pH measured within the biofilms was about 0.6 units lower. In the cultures without pH control, the *in situ* pH of the biofilms was slightly higher than the pH measured in the fluid phase, probably due to the cells acting as an effective buffer, and 0.8 pH units lower than in the biofilms formed with buffered medium. Therefore, the use of buffered medium was again an effective means to generate populations of biofilm cells exposed to a significant difference in pH, and the effects of pH on exopolysaccharide gene expression could be reliably examined.

CAT specific activity in biofilm cells at 'quasi-steady state' and following a carbohydrate pulse

Biofilm cells were cultivated in a sucrose-containing medium to quasi-steady state at a dilution rate of 0.1 h^{-1} for a minimum of a calculated 10 generations, i.e. >69 h (Burne & Chen, 1998). CAT activities were measured immediately before addition of sucrose to a final concentration of 25 mM and at 15, 30 and 60 min after the addition of sucrose (Table 3). Addition of sucrose in all cases resulted in a steady decline in the pH of the planktonic phase (Table 3). At quasi-steady state, CAT activity in the SMS101 (*ftf-cat*) strain that was cultivated at the lower pH was always modestly lower than in biofilm cells grown with pH control, whereas SMS102 (*gtfBC-cat*) showed about a twofold increase of CAT when grown at lower pH values. Expression of the *ftf* and *gtfBC* genes was induced more than two- and fourfold, respectively, by the addition of sucrose to the steady-state cultures grown in buffered medium. An increase in CAT activity of about threefold in both

Table 1. Physical characteristics of the biofilms of recombinant *S. mutans* SMS101 growing in sucrose-defined media at quasi-steady state

Culture conditions	Dry weight ($\mu\text{g cm}^{-2}$)	$10^{-8} \times$ Direct counts (cm^{-2})*	$10^{-8} \times$ Viable counts (cm^{-2})†	Total carbohydrate (%)
pH control‡	180 ± 24	2.65 ± 0.25	1.98 ± 0.28	49
No pH control§	277 ± 40	6.24 ± 0.95	3.92 ± 0.50	68

* Direct counts were determined in a Petroff–Hauser counter as detailed in Methods.

† Viable counts determined by plating on BHI with $10 \mu\text{g}$ erythromycin ml^{-1} .

‡ Biofilms cultivated in media buffered with 50 mM potassium phosphate buffer (pH 7.8).

§ Biofilms cultivated in medium containing 90 mM KCl.

Table 2. *In situ* pH profiles in the biofilms of recombinant *S. mutans* strains growing in sucrose-defined media at quasi-steady state

Strain	With pH control*		Without pH control†	
	pH in fluid phase	<i>In situ</i> pH	pH in fluid phase	<i>In situ</i> pH
SMS101	6.68 ± 0.15	6.08 ± 0.12	5.12 ± 0.25	5.32 ± 0.10
SMS102	6.72 ± 0.12	6.11 ± 0.12	5.14 ± 0.16	5.25 ± 0.14

* Biofilms cultivated in media buffered with 50 mM potassium phosphate buffer (pH 7.8).

† Biofilms cultivated in medium containing 90 mM KCl.

Table 3. CAT specific activity of recombinant *S. mutans* strains growing in *in vitro* biofilms under sucrose limitation and following a 25 mM sucrose pulse

Quasi-steady-state biofilms were formed for 10 generations with sucrose as the limiting carbohydrate prior to addition of sucrose to the vessel, as detailed in the text.

Time (min) after sucrose pulse	Strain SMS101 (<i>ftf-cat</i>)		Strain SMS102 (<i>gtfBC-cat</i>)	
	pH after sucrose pulse	CAT activity [$\text{U (mg protein)}^{-1}$]*	pH after sucrose pulse	CAT activity [$\text{U (mg protein)}^{-1}$]*
With pH control‡				
T_0	6.68 ± 0.15	0.112 ± 0.08	6.72 ± 0.12	0.054 ± 0.03
T_{15}	6.34 ± 0.13	0.265 ± 0.10	5.76 ± 0.10	0.112 ± 0.04
T_{30}	5.87 ± 0.12	0.290 ± 0.08	5.21 ± 0.11	0.185 ± 0.06
T_{60}	5.51 ± 0.08	0.269 ± 0.05	4.98 ± 0.07	0.232 ± 0.12
Without pH control‡				
T_0	5.12 ± 0.25	0.091 ± 0.05	5.14 ± 0.16	0.106 ± 0.04
T_{15}	4.90 ± 0.33	0.298 ± 0.08	4.91 ± 0.14	0.224 ± 0.08
T_{30}	4.74 ± 0.28	0.262 ± 0.11	4.70 ± 0.04	0.318 ± 0.07
T_{60}	4.63 ± 0.35	0.183 ± 0.12	4.58 ± 0.06	0.382 ± 0.10

* Units are defined as μmol chloramphenicol acetylated min^{-1} ($\text{mg protein})^{-1}$. The values shown are means from at least three separate Rotatorque runs and all measurements were acquired in at least triplicate.

† Biofilms cultivated in media buffered with 50 mM potassium phosphate buffer (pH 7.8).

‡ Biofilms cultivated in medium containing 90 mM KCl.

Table 4. CAT specific activity of recombinant *S. mutans* strains growing in *in vitro* biofilms with glucose limitation following a 25 mM sucrose pulse

Biofilms were formed for 10 generations with sucrose as the limiting carbohydrate, then quasi-steady state was established under glucose-limiting conditions prior to addition of sucrose to the vessel, as detailed in the text.

Time (min) after sucrose pulse	Strain SMS101 (<i>ftf-cat</i>)		Strain SMS102 (<i>gtfBC-cat</i>)	
	pH after sucrose pulse	CAT activity [U (mg protein) ⁻¹]*	pH after sucrose pulse	CAT activity [U (mg protein) ⁻¹]*
With pH control†				
<i>T</i> ₀	6.62 ± 0.12	0.088 ± 0.07	6.64 ± 0.15	0.042 ± 0.04
<i>T</i> ₁₅	6.38 ± 0.18	0.226 ± 0.12	6.20 ± 0.22	0.176 ± 0.08
<i>T</i> ₃₀	5.92 ± 0.13	0.287 ± 0.10	5.80 ± 0.21	0.258 ± 0.05
<i>T</i> ₆₀	5.52 ± 0.24	0.254 ± 0.09	5.49 ± 0.18	0.295 ± 0.06
Without pH control‡				
<i>T</i> ₀	5.08 ± 0.22	0.098 ± 0.07	5.11 ± 0.25	0.082 ± 0.03
<i>T</i> ₁₅	4.89 ± 0.28	0.326 ± 0.14	4.92 ± 0.22	0.214 ± 0.07
<i>T</i> ₃₀	4.82 ± 0.33	0.268 ± 0.12	4.80 ± 0.19	0.342 ± 0.12
<i>T</i> ₆₀	4.72 ± 0.24	0.154 ± 0.12	4.79 ± 0.23	0.395 ± 0.14

* Units are defined as μmol chloramphenicol acetylated min⁻¹ (mg protein)⁻¹. The values shown are means from at least three separate Rotatorque runs and all measurements were acquired in at least triplicate.

† Biofilms cultivated in media buffered with 50 mM potassium phosphate buffer (pH 7.8).

‡ Biofilms cultivated in medium containing 90 mM KCl.

Table 5. CAT specific activity of recombinant *S. mutans* strains growing in *in vitro* biofilms under sucrose limitation and following a 25 mM glucose pulse

Quasi-steady-state biofilms were formed for 10 generations with sucrose as the limiting carbohydrate prior to addition of glucose to the vessel, as detailed in the text.

Time (min) after glucose pulse	Strain SMS101 (<i>ftf-cat</i>)		Strain SMS102 (<i>gtfBC-cat</i>)	
	pH after glucose pulse	CAT activity [U (mg protein) ⁻¹]*	pH after glucose pulse	CAT activity [U (mg protein) ⁻¹]*
With pH control†				
<i>T</i> ₀	6.70 ± 0.15	0.103 ± 0.03	6.65 ± 0.15	0.038 ± 0.03
<i>T</i> ₁₅	6.20 ± 0.20	0.124 ± 0.05	6.20 ± 0.12	0.076 ± 0.04
<i>T</i> ₃₀	5.80 ± 0.15	0.118 ± 0.04	5.75 ± 0.05	0.115 ± 0.04
<i>T</i> ₆₀	5.50 ± 0.20	0.125 ± 0.06	5.50 ± 0.10	0.126 ± 0.06
Without pH control‡				
<i>T</i> ₀	5.04 ± 0.20	0.084 ± 0.02	5.12 ± 0.18	0.114 ± 0.03
<i>T</i> ₁₅	4.82 ± 0.25	0.188 ± 0.05	4.84 ± 0.22	0.167 ± 0.05
<i>T</i> ₃₀	4.65 ± 0.26	0.175 ± 0.04	4.66 ± 0.20	0.198 ± 0.07
<i>T</i> ₆₀	4.60 ± 0.24	0.116 ± 0.03	4.62 ± 0.21	0.224 ± 0.06

* Units are defined as μmol chloramphenicol acetylated min⁻¹ (mg protein)⁻¹. The values shown are means from at least three separate Rotatorque runs and all measurements were acquired in at least triplicate.

† Biofilms cultivated in media buffered with 50 mM potassium phosphate buffer (pH 7.8).

‡ Biofilms cultivated in medium containing 90 mM KCl.

SMS101 and 102 grown without pH control was seen after addition of sucrose. In all cases, there were differences in the kinetics of the induction between the *gtf* and *ftf* gene fusions and the induction pattern was

different depending on whether the strains were cultivated with pH control or not. CAT activity in the *gtf* gene fusion strain increased steadily over a 1 h period following sucrose addition to biofilms grown in

unbuffered medium. In contrast to the behaviour of SMS102, the induction of the *ftf* gene fusion in SMS101 by sucrose occurred within 15 min and was generally followed by a decline in CAT, the rate of which depended on the growth conditions. Notably, the rate of decline of CAT activity in SMS101 following a sucrose pulse was slower than that previously reported for strain SMS101 in biofilms (Burne *et al.*, 1997), perhaps because of differences in the composition of the culture media or the time allowed for formation of biofilms in this study.

To determine whether the limiting growth carbohydrate in steady-state biofilms could affect the expression of *gtf* or *ftf*, biofilms were formed in the presence of sucrose for 10 vessel volume changes, and then for an additional 10 vessel volume changes in the base medium containing 5 mM glucose. When biofilms were formed in buffered medium, the level of CAT activity expressed by these strains at quasi-steady state, before addition of sucrose, was essentially the same as that observed for biofilms growing with sucrose as the limiting carbohydrate. After addition of 25 mM sucrose to the glucose-limited biofilms, the patterns for induction of *ftf* and *gtfBC* genes were essentially similar to those patterns observed when quasi-steady-state, sucrose-limited biofilms were pulsed with sucrose (Table 4). Repeating this experimental strategy with glucose-limited biofilms formed in the absence of pH control (Table 4) yielded results essentially identical to those seen with biofilms formed in sucrose (Table 3). In all cases then, and in contrast to when cultures were exposed to excess sucrose, growth with sucrose as the limiting carbohydrate did not result in higher *gtf* or *ftf* expression when compared with glucose-limited biofilms. Moreover, growth in limiting concentrations of glucose did not change the ability of the strains to respond to induction by sucrose.

Finally, quasi-steady-state biofilms were formed in a sucrose-containing medium followed by a 25 mM glucose pulse (Table 5). Interestingly, the CAT specific activity of strain SMS102 (*gtfBC-cat*) increased about fourfold when glucose was added to biofilms growing in buffered medium. CAT specific activities of strain SMS102 also increased about twofold when glucose was added to biofilms growing without pH control. In the case of *S. mutans* SMS101 growing with pH control, there was little increase in the level of CAT activity after addition of glucose to the vessel. However, in SMS101 biofilms grown without pH control, transcription of *ftf* increased a little more than twofold after glucose addition, and then rapidly declined to baseline levels within 1 h.

DISCUSSION

S. mutans in dental biofilms is subjected to a variety of different environmental conditions, most notably sudden changes in the type and amount of nutrients, accompanied by rapid and significant fluctuations in pH (Burne, 1998a; Carlsson, 1984). The ability of this organism to sense and respond to changes in the

environment is crucial to its survival and cariogenicity (Bowden & Hamilton, 1998). In a previous study, significant differences in expression of *gtf* and *ftf* were observed in mature biofilms versus relatively thinner biofilms or planktonic cells (Burne *et al.*, 1997). One possible explanation for the differences in expression of the exopolysaccharide-synthesizing machinery of *S. mutans* in thicker biofilms is that, because of mass transport limitations, the pH and carbohydrate concentrations experienced by the organisms in the thick biofilms differ significantly from those to which planktonic cells or thinner biofilms are exposed. Results presented in this communication support the idea that expression of the *ftf* and *gtfBC* genes in biofilms is strongly influenced by carbohydrate availability, the type of carbohydrate, and the environmental pH.

A number of new findings have emerged from this study. The first is that environmental pH clearly influences the expression of the *gtfBC* genes of *S. mutans* growing in biofilms. Biofilms grown without pH control, which had biofilm pH values of around 5.3, consistently had twofold more CAT activity expressed from the *gtfBC* promoter than SMS102 cultivated with pH control. Moreover, following a carbohydrate pulse, the level of expression of *gtfBC* in the biofilms without pH control was significantly higher than in biofilms with pH control. Since increased production of GtfBC would likely result in a greater proportion of carbohydrate being incorporated into biofilm polysaccharides, this observation is consistent with the finding that the biofilms formed at low pH were composed of a much greater percentage of carbohydrate than those formed at a more neutral pH value. Also, a previous study (Wexler *et al.*, 1993) showed that when *S. mutans* was grown in steady-state continuous chemostat culture, lowering of the pH from 7 to 6 resulted in about a 50% increase in the expression of the *gtfBC* genes. Interestingly, the level of expression of *gtfBC* achieved by suspended populations at steady state or after a sucrose pulse (Wexler *et al.*, 1993) was lower than the level of expression seen in biofilms at quasi-steady state or after sucrose induction in this study. Thus, one possible explanation for the enhanced *gtfBC* expression in biofilms may be due to the relatively lower pH values achieved in biofilms (Table 2), compared to the continuous culture studies, or to the existence of low pH microenvironments in which *gtf* expression is markedly elevated. A second significant, and possibly related, finding is that *gtfBC* expression in biofilms was induced by addition of excess glucose to the culture, albeit the level of induction was not as great as that seen with sucrose. There is no logical reason for glucose, which is not a substrate for Gtfs, to act as a specific inducer of *gtfBC*. Instead, it is more likely that the *gtfBC* genes are induced in response to acidification of the biofilms or in response to the presence of an excess of a metabolizable carbohydrate. The latter could be signalled by directly sensing carbohydrate flow through the PTS or the glycolytic pathway, or could reflect a response to increased growth rate, although in continuous culture we found *gtfBC* expression was inversely

correlated with how fast the cells were growing (Wexler *et al.*, 1993). Of note, there does not seem to be a hierarchy of control of expression of *gtfBC* by carbohydrate and pH, since induction by excess carbohydrate occurs whether the cells are growing with or without pH control. Possibly, then, the effects of pH and carbohydrate are exerted through different pathways. Finally, we cannot exclude the idea that differences in phosphate concentrations in the buffered and unbuffered cultures also had an impact on the results.

Another important finding was that growth of the biofilms under quasi-steady-state conditions with glucose as the limiting carbohydrate did not result in down-regulation of *gtf* or *ftf* expression compared to cells growing with sucrose as the limiting carbohydrate. It has been suggested that sucrose is a specific inducer of exopolysaccharide-producing enzymes, but if that were the case, then one might expect higher levels of CAT activity expressed at T_0 in cells growing in sucrose (Tables 3 and 5) versus CAT activities at T_0 in biofilms being fed on glucose (Table 4), which was not the case. It could be that high levels of sucrose are required for induction, although this would not explain why both *ftf* and *gtf* can be induced by addition of either glucose or sucrose. These findings emphasize the possibility that lowering of the pH following addition of carbohydrates or that an increase in the availability of carbohydrate, perhaps sensed via the PTS or glycolytic intermediates, are mechanisms by which *gtf* and *ftf* gene expression could be regulated. In fact, we now have preliminary evidence (unpublished) from continuous chemostat culture of these strains at different pH values, under carbohydrate-limiting and carbohydrate-excess conditions, that not only pH, but the amount of carbohydrate available to cells is a factor that influences both *ftf* and *gtf* gene expression.

The expression pattern of *ftf* is more complex than that of *gtfBC*. As with *gtf*, the expression levels of *ftf* in quasi-steady-state biofilms are the same regardless of whether glucose or sucrose was the limiting carbohydrate. In contrast, *ftf* expression in the lower pH biofilms was not enhanced above that in biofilms formed with buffered medium. As seen previously with both suspended (Wexler *et al.*, 1993) and adherent (Burne *et al.*, 1997) populations of *S. mutans*, sucrose was an efficient inducer of *ftf* gene expression. In this study, we found that induction by sucrose occurred regardless of whether the cells were growing at the lower or higher pH value, or whether the steady-state biofilms were formed with glucose or sucrose as the limiting carbohydrate. However, addition of glucose to quasi-steady-state biofilms had only a modest effect on induction of *ftf*. In continuous chemostat culture, increasing the growth rate of strain SMS101, or lowering of the pH from 7 to 6, enhanced *ftf* transcription (Wexler *et al.*, 1993), so these factors were likely to have had an effect on *ftf* transcription after addition of excess carbohydrate. Still, in all cases, the responses of *ftf* to added carbohydrate, i.e. the rate of induction and the subsequent decline in CAT activity, differed as a function of

whether the culture medium was buffered or not. Thus, global factors regulating gene expression in relation to carbohydrate flow or pH may overlap with the regulatory networks that control *ftf* transcription.

Another notable finding is that expression from the *gtfBC* promoter in biofilms in this study was higher than that observed in cells grown in continuous chemostat culture (Wexler *et al.*, 1993), although not as high as in mature (7 d) biofilms (Burne *et al.*, 1997). For example, values in the chemostat were reported to range from 0.017 to 0.049 U (mg protein)⁻¹ under steady-state conditions, and levels after sucrose induction were as high as 0.15 U (mg protein)⁻¹. It can be inferred from the results obtained here that the existence of low pH microenvironments and perhaps the presence of extracellular storage polysaccharides that increase the amount of carbohydrate available to subpopulations of cells within the biofilm may account for the apparent stimulation of *gtf* expression in biofilms (Burne *et al.*, 1997). Low growth rate microenvironments could also exist and impact *gtfBC* expression. We also have noted that *ftf* expression in mature (7 d) biofilms is almost completely repressed. Interestingly, the level of CAT expressed from the *ftf* gene fusion in quasi-steady-state biofilms (Tables 3–5) was about one-third of that in suspended cells in steady-state continuous chemostat culture (Wexler *et al.*, 1993). Also, following induction of *ftf* in the chemostat using sucrose, levels of CAT peaked at about 0.45 U (Wexler *et al.*, 1993), whereas the peak expression noted here was about 0.33 U. So again, the trend is that *ftf* expression in biofilms is partially repressed compared with suspended populations. Recently, we have also found evidence that growth at pH 5 leads to nearly complete repression of *ftf* (C. Browngardt & R. A. Burne, unpublished), so low pH in the biofilms may account in part for lowered expression of *ftf* in biofilms.

Clearly, pH and the source and amount of carbohydrate influence the transcription of the exopolysaccharide machinery of *S. mutans* in biofilms. However, a number of other factors could also account for altered *gtf* and *ftf* expression in mature biofilms. The first is that the availability of other nutrients and oxygen may be altered compared to young biofilms or planktonic cells. Goodman & Gao (2000) have found that *gtf* expression occurs at specific growth phases and have suggested a possible cell-density-dependent component regulating *gtfBC*, raising the possibility that intercellular signalling may be involved in regulating *gtf* expression. Although the results we have collected thus far do not fully support that quorum sensing is a regulator of exopolysaccharide synthesis in *S. mutans*, a *luxS* homologue is identifiable in the *S. mutans* chromosome, and peptide-mediated intercellular signalling is also established in *S. mutans* (Li *et al.*, 2001). Therefore, the idea that exopolysaccharide production is regulated by quorum sensing mechanisms cannot yet be excluded. In summary, this study highlights the need for additional research with suspended and adherent oral streptococci to determine whether the changes in the expression

patterns of genes of *S. mutans* growing in biofilms are restricted to spatially isolated subpopulations or to pathways that globally regulate biofilm gene expression in densely packed populations.

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