Streptococcus mutans Fructosyltransferase (tf) and Glucosyltransferase (gtfBC) Operon Fusion Strains in Continuous Culture

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Three glucosyltransferases (GTFs), which catalyze the formation of water-insoluble adherent glucans, and fructosyltransferase (FTF), which synthesizes fructans, are believed to contribute to the pathogenic potential of Streptococcus mutans. Study of the regulation of expression of GTF and FTF has been difficult because of the complexity and number of exoenzymes produced by this bacterium. By using continuous chemostat culture to control environmental conditions, chloramphenicol acetyltransferase (CAT) operon fusions were utilized to measure transcriptional activity of the tf and gtfBC gene promoters. Expression of these operon fusions was differentially regulated in response to culture pH and growth rate and during transition states between growth domains. Furthermore, the addition of sucrose to steady-state cultures resulted in significant increases in CAT specific activities for both fusions. In a few cases, GTF and FTF enzyme specific activities did not parallel those of the corresponding CAT fusion activities; this lack of correspondence was likely due to posttranscriptional events controlling enzyme secretion and enzyme activity, as well as to the differential expression of dextranase(s) and fructan hydrolase by S. mutans. These results clearly demonstrate that the extracellular polymer synthesis machinery of S. mutans is regulated in a complex manner. The use of operon fusions in combination with chemostat culture is a viable approach to analyzing gene expression in S. mutans and will be helpful in defining the molecular mechanisms underlying regulation of expression of virulence attributes under conditions that may more closely mimic those in dental plaque.

The virulence of Streptococcus mutans is dependent on several gene products and factors which contribute, to varying degrees, to the pathogenic potential of this organism. Among these virulence attributes, the ability to synthesize extracellular polysaccharides from sucrose has been demonstrated to augment the cariogenicity of oral streptococci (15, 18). S. mutans synthesizes glucans and fructans from sucrose via the actions of glucosyltransferases (GTFs) and a fructosyltransferase (FTF), respectively. Glucans appear to serve in multiple capacities, including mediating initial adherence to the tooth surface (39, 45), facilitating bacterial accumulation on smooth surfaces (13, 32), and as a reservoir for metabolizable polysaccharides outside of the cell (5). Fructans are believed to function exclusively as extracellular storage compounds (5, 33, 58), but few investigations into other roles for this abundant plaque constituent have been conducted.

S. mutans produces at least three GTFs. GTF-S is the product of the gtfD gene and catalyzes the formation of a relatively water-soluble glucan composed almost entirely of α(1,6) linkages (23). The GTF-I and GTF-SI enzymes, the products of the gtfB and gtfC genes, respectively, synthesize predominantly water-insoluble glucans. These polysaccharides are rich in α(1,3) linkages but also contain substantial amounts of α(1,6)-glucan (1, 22). Previous studies (51) have indicated that the gtfB and gtfC genes are cotranscribed from a single promoter. The gtfD gene is not genetically linked to the gtfBC locus (37). In the rat caries model, strains defective in the synthesis of active gtfBC gene products demonstrated marked reductions in the ability to elicit smooth-surface caries (36, 44), while inactivation of GTF-S appeared to have little effect on cariogenicity (36).

S. mutans produces a single FTF, which catalyzes the synthesis of fructans composed predominantly of β(2,1) linkages (3, 11). Plaque fructans accumulate rapidly in vivo following ingestion of sucrose (16) and are then hydrolyzed to fructose by fructan hydrolases produced by S. mutans (6, 9, 53) and other oral bacteria (49). A significant contribution of FTF, plaque fructans, and fructan hydrolases to virulence has not been readily demonstrable by using otherwise-isogenic mutants in the rat caries model (30, 57). However, experiments to allow for the manifestation of the full cariogenic potential of organisms which can utilize storage polysaccharides have not been performed.

The use of a chemostat to modulate the growth rate, pH, and other culture conditions has illustrated the remarkable phenotypic plasticity of the oral streptococci. By altering the growth environment, a wide range of phenotypic changes in polymer synthesis capacities (12, 55, 56), adherence capabilities (40), acidogenicity (27), acidurance (2, 20), carbohydrate transport (19), and glycolytic capacities (7) of S. mutans has been elicited. In particular, it has been demonstrated that the phenotypic expression of the glucan and fructan synthetic capacities of oral streptococci is regulated in response to multiple environmental variables (24, 34, 50). However, little is known about the expression of individual gene products participating in polysaccharide metabolism. This is largely due to the fact that a variety of factors have complicated the study of individual enzymes involved in polysaccharide synthesis. For example, the antigenic similarity between GTFs (31, 43, 48) and the breakdown of GTFs
mutans strains either laboratories, Detroit, Mich.) on maintained or allowed to grow for a common substrate, which may influence measurable activities. Other factors, such as association of lipoteichoic acids, which are found in large quantities in the culture supernates under particular conditions and can associate with glucans, may affect the way in which the polysaccharide is precipitated, since lipoteichoic acids are amphipathic. Also, the presence of differentially regulated glucan and fructan hydrolases (26, 54), which attack the products of the GTFs and FTF, will influence the amounts of total polysaccharide measured in biochemical reactions. Undoubtedly, other factors, such as proteases or enzyme inhibitors in cell supernates, could influence measurable activity. Clearly then, a combination of methods will be necessary to completely dissect the molecular basis for the general observation that the phenotypic expression of polysaccharide synthesis is differentially regulated in response to environmental conditions.

In order to better understand the modes and mechanisms of regulation of expression of gtfBC and fff, chloramphenicol acetyltransferase (CAT) fusions in conjunction with chemostat culture were utilized to monitor the transcriptional activity from the promoters of these genes as a function of culture conditions. The fusions were constructed in such a manner as not to disrupt the normal gtf or fff genes (24). This technique allowed for the measurement of transcriptional activity from the gtfBC and fff promoters and of extracellular polymer synthesis as a function of growth rate and pH and in response to the addition of sucrose to steady-state cultures.

(A portion of this data was presented at the 1992nd General Meeting of the American Society for Microbiology, 26–30 May 1992, New Orleans, La. [56a].)

MATERIALS AND METHODS

Bacterial strains and media. S. mutans SMS101 (fff-cat) and SMS102 (gtfBC-cat) have been previously described (24). The chromosomal structures of these strains at the fff or gtfBC locus are shown in Fig. 1. These strains were maintained on brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of erythromycin per ml. For continuous culture experiments, S. mutans strains were grown in a Bio-Flo III chemostat (New Brunswick Scientific, Edison, N.J.) in tryptone-yeast extract (TY; Difco) medium (6) supplemented with 10 mM glucose and 10 μg of erythromycin per ml.

Growth conditions and preparation of enzymes. One liter of TY broth was inoculated with 1 ml of a TY starter culture of either S. mutans SMS101 or S. mutans SMS102 and grown in the Bio-Flo III at a dilution rate (D) of 0.05 h⁻¹. D is defined as the amount of medium added as a fraction of the total starting vessel volume per unit of time (e.g., D = 0.05 h⁻¹ indicates that a 0.05 vessel volume, 50 ml, was added to the chemostat vessel, which was held at a constant volume of 1 liter, per hour). Steady-state growth was assumed when the culture was maintained for at least 10 generations under a particular growth condition. Control of culture pH was accomplished by the addition of 2.0 M potassium hydroxide. For sucrose induction experiments, a 0.75 M sucrose solution was added to the vessel to yield a final concentration of 20 mM sucrose. To assess the stability of the operon fusions, samples of the culture at the end of an experiment were diluted and plated on BHI and on BHI with 10 μg of erythromycin per ml. Counts (CFU) on BHI and BHI plus erythromycin were compared to evaluate the stability of the Em' marker. Similarly, CAT levels at the beginning and end of each experiment, under identical conditions, were compared since lower enzyme activity following long-term culture would be indicative of loss of the CAT fusion.

To sample the culture, 50-ml aliquots were removed at prescheduled times and immediately centrifuged at 8,000 × g for 10 min at 4°C. The supernate was dialyzed against 10 mM potassium phosphate buffer, pH 6.0, containing 1 mM phenylmethylsulfonfluoride (PMSF; Sigma Chemical Co., St. Louis, Mo.) and 0.02% sodium azide (Sigma). The dialysate was utilized for determining extracellular enzyme activity. The cell pellet was quickly frozen in a dry-ice–ethanol bath and stored at −70°C. For determination of CAT specific activity, the pellet was resuspended in 1.0 ml of 10 mM Tris-HCl buffer, pH 7.8, containing 10 mM hexanoyl acid (Sigma). A one-third volume of glass beads (average diameter, 0.2 mm; Baxter Scientific) was added, and the cells were disrupted by homogenization with a Tissue Tearor cell homogenizer (Biospec Products Inc., Bartlesville, Okla.) at maximum speed for 8 min with intermittent CO₂-ethanol cooling. The lysates were centrifuged at 12,000 × g, and the supernate was used to determine CAT-specific activities (see below).

Enzymatic assays. The determination of CAT specific activities was accomplished by the spectrophotometric method of Shaw (47), which measures the rate of acetylation of chloramphenicol. Coincident with the transfer of an acetyl group from acetyl coenzyme A to chloramphenicol is the reaction of the reduced coenzyme A with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). One unit of CAT activity is equivalent to 1 μmol of chloramphenicol acetylated per min.
Protein concentrations were determined by the method of Bradford (4), by using a commercially available kit (Bio-Rad), with bovine serum albumin as the standard.

Culture supernatants from SMS101 and SMS102 treated as described above were assayed for FTF and GTF activity by a modification of the method of Robrish et al. (38) and Germaine et al. (14), respectively. Briefly, for FTF, the assay determined the incorporation of the fructose moiety from [fructose-1-3H(N)]-sucrose into methanol-insoluble polysaccharides. The enzyme preparations were incubated with an equal volume of the substrate mixture containing 10 mM potassium phosphate buffer (pH 6.0), 0.02% sodium azide, sucrose (200 mM), and 100 mM [fructose-1-3H(N)]-sucrose. After incubation for 4 h at 37°C, 1 ml of ice-cold methanol was added. The precipitated polysaccharides were washed with ice-cold methanol in a filter manifold (Millipore Corporation, Bedford, Mass.) onto a 2.5-cm-diameter glass fiber filter (Whatman). The filters were dried and immersed in scintillation fluid (Ecocint A; National Diagnostics, Manville, N.J.). The amount of radiolabelled sugar incorporated into the polysaccharides was measured by scintillation counting. GTF assays were performed essentially as described for FTF, except that [glucose-14C(U)]-sucrose, 40 μM dextran 9000, and 0.04 M Imidazole-HCl buffer (pH 6.5) were used in the substrate mixture instead of [fructose-1-3H(N)]-sucrose and phosphate buffer. One unit of GTF or FTF activity was defined as the amount of enzyme necessary to incorporate 1 μmol of hexose into methanol-insoluble material in 1 min. All assays were performed in triplicate. All data were collected from three separate chemostat runs and are the averages of the results of triplicate assays. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard.

RESULTS

Steady-state growth experiments. Growth rate and pH have been previously demonstrated to modulate glucan and fructan synthetic capabilities of S. mutans growing at steady state (28, 56). To determine the influence that growth at low (0.05 h−1; doubling time, 13.8 h) and high (0.3 h−1; doubling time, 2.3 h) dilution rates and environmental pH (pH 6.0 or 7.0) had on transcription from the gtf and ftf promoters and on exoenzyme activity, cultures were grown to steady state at each set of conditions. The vessel was sampled, and CAT, GTF, and FTF specific activities were determined (Table 1). Since the gtfBC and ftf structural genes were not disrupted in the construction of the fusion strains, it was possible to determine FTF or GTF activity. In all cases, at steady state, the organisms were growing under glucose-limiting conditions. No residual glucose was detected in the culture supernates by using a glucose oxidase reaction (Sigma Chemical Co.) (data not shown), indicating that less than 5 μM glucose remained in the vessel.

For SMS101(ftf-cat), transcription from the ftf promoter as determined by CAT-specific activity (CAT-fft) was not significantly different between dilution rates of 0.05 and 0.3 h−1 when sampled at pH 6.0. However, when sampled at pH 7.0, there was a twofold increase in CAT-fft noted for cells grown at the faster growth rate. In all cases, CAT-fft was higher in cells grown at pH 6.0 than at pH 7.0. No differences were observed in FTF activity as a function of the growth rate at pH 6.0. However, at pH 7.0 and D = 0.3 h−1, measurable extracellular FTF specific activity was some fivefold lower than that observed for cells grown at pH 7.0 and D = 0.05 h−1, despite the fact that CAT-fft was doubled at the faster growth rate. Likewise, FTF specific activity was significantly higher in culture supernates of pH 7.0-grown cells than in those of pH 6.0-grown cells, under circumstances at which transcription from the ftf promoter was much greater for cells cultured at pH 6.0. Clearly, the culture pH and the specific growth rates of the organisms influenced transcription rates from the ftf promoter, as well as phenotypic expression of extracellular FTF activity. Further, the influence of growth rate on the cells occurred in a pH-dependent fashion.

In SMS102(gtfB-cat), transcription from the gtfBC promoter as measured by CAT-specific activity (CAT-gtf) was consistently lower for cells grown at a dilution rate of 0.3 h−1 than of 0.05 h−1 regardless of the culture pH. Extracellular GTF specific activity differed only slightly between the two steady-state levels and between pH values. Under all steady-state conditions, extracellular GTF specific activity was not significantly diminished at the higher dilution rates despite measurable decreases in CAT-gtf.

CAT fusion and extracellular enzyme activities during transition states between steady-state growth domains. The levels of CAT and polymer synthesis activities from S. mutans SMS101 and SMS102 were determined during a shift up to a high growth rate (steady-state D = 0.05 h−1 to D = 0.4 h−1) and a shift down to a low growth rate (steady-state D = 0.3 h−1 to D = 0.05 h−1) at a constant pH value of either 6.0 or 7.0. Cultures could not be maintained without washout at D = 0.4 h−1, likely because of the glucose limitation. Responses by the organisms to alterations in the dilution rates occurred for both fusion strains, and the patterns of CAT- and exoenzyme-specific activities observed for each strain during the transition states differed from those observed at steady state for the culture pH. It should be noted that during the shift in growth

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<td>6.0</td>
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* Values are the averages for one of three separate chemostat runs. Activities are expressed as units per minute per milligram of protein ± the standard deviations of triplicate samples. Steady-state dilution rates of 0.05 and 0.3 h−1 were reached after cultures were allowed to grow for 10 generations at each dilution rate.
rate from $D = 0.05 \, \text{h}^{-1}$ to $0.4 \, \text{h}^{-1}$, the cultures were not glucose limited.

At pH 7.0, CAT-ff increased dramatically with the increase in the dilution rate in the vessel (Fig. 2A) after approximately 1 h following the shift. Extracellular FTF activity, on the other hand, decreased dramatically during this period (Fig. 2A), consistent with the lower activity noted for cells growing at steady state at pH 7.0, $D = 0.3 \, \text{h}^{-1}$ compared with $D = 0.05 \, \text{h}^{-1}$ (Table 1). It seems unlikely that this phenomenon was simply due to enzyme washout, since specific activities and not units of activity per milliliter were recorded. Also, there was not sufficient dilution of the culture during the transition to account for the decrease in specific activity. Moreover, the decrease occurred in a discontinuous manner, with most of the reduction during the first hour of the transition.

Following reequilibration of the vessel and establishment of steady-state growth at $D = 0.3 \, \text{h}^{-1}$ (10 generations), the dilution rate was shifted from $0.3 \, \text{h}^{-1}$ to $0.05 \, \text{h}^{-1}$ (Fig. 2B). Following the shift down, an increase in CAT-ff and extracellular FTF specific activities was noted, with a subsequent decline in both activities after 30 min.

When these experiments were performed with SMS101 at pH 6.0 (Fig. 3), a pattern different from that for pH 7.0 was observed. Following shift up (Fig. 3A), a decrease in CAT-ff and a small decrease in extracellular FTF specific activity were noted. After the vessel had equilibrated and cells had been cultured for 10 generations at $D = 0.3 \, \text{h}^{-1}$ and pH 6.0, the dilution rate was reduced to $0.05 \, \text{h}^{-1}$ and the vessel was sampled (Fig. 3B). In this case, slight decreases in CAT-ff and FTF specific activities were observed, and no additional decrease following 1 h at the new dilution rate was seen.

The identical manipulations of the growth conditions for a transition-state culture were performed with $S. \ mutans$ SMS102($ggtBC$-cat), and CAT-gtf and extracellular GTF specific activities were measured (Fig. 4 and 5). At pH 7.0, an increase in GTF-specific activity was noted upon shift up, followed by a decline in this activity within 1 h (Fig. 4A). This increase in GTF activity was not accompanied by a coincident increase in CAT-gtf, but rather a steady decline in
CAT-gtf activity was noted during the shift up. After steady state was achieved at $D = 0.3 \text{ h}^{-1}$, the dilution rate was adjusted to $0.05 \text{ h}^{-1}$ (Fig. 4B). This shift was accompanied by only slight changes in CAT-gtf and glucan synthesis activities.

At pH 6.0, a decline in both CAT-gtf and GTF specific activities was noted when the dilution rate was increased (Fig. 5A). Alternatively, adjustment of the dilution rate from 0.3 to $0.05 \text{ h}^{-1}$ was accompanied by an increase in assayable GTF specific activity but with little change in the expression from the CAT fusion (Fig. 5B). GTF specific activity declined to steady-state levels within 2 h of the shift to $0.05 \text{ h}^{-1}$. Again, the values measured for CAT and GTF approached those recorded for steady-state-grown cells fairly rapidly (Table 1).

**Responses of low-growth-rate cultures to sucrose.** The effects of the addition of sucrose to cultures of glucose-limited *S. mutans* growing at a low growth rate on transcriptional fusion activity and on polymer synthesis were examined as detailed in Materials and Methods. The addition of sucrose (final concentration, 20 mM) to *S. mutans* SMS101(ffl-cat) resulted in a rapid increase in CAT-ffl of approximately 50\% for 40 min after the introduction of sucrose (Fig. 6). This increase was followed by a slow decline in CAT-ffl. In contrast, only a slight increase in FTF specific activity occurred for 30 min after the sucrose addition, followed by a decline in the assayable activity.

The baseline levels of CAT-gtf were significantly lower than those expressed with the ffl-cat fusion strain, consistent with observations for steady-state low-growth-rate cultures (Table 1). The addition of sucrose to slowly growing glucose-limited SMS102(gtfBC-cat) cultures resulted in a threefold increase in expression from the gtfBC promoter as measured by CAT-gtf (Fig. 7). There was a phenotypic lag in expression of extracellular GTF specific activity after the sucrose addition for at least 10 min, during which there was actually a slight but consistently repeatable decrease in assayable glucan synthesis, perhaps reflecting the aggregation of an existing supernatant enzyme by nascent dextrans. The lag period was followed by an increase of approximately 40\% in extracellular GTF activity over 90-min. Unlike that for FTF, no decrease was seen in extracellular GTF specific activity during these experiments.

**Stability of CAT fusions.** A single chemostat run for each fusion strain lasted 4 to 5 weeks, approximately 60 to 80 generations. The effect that long-term continuous culture would have on the stability of the fusions was not known, even though erythromycin was included in the culture me-

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**FIG. 4.** CAT and extracellular GTF activities as a measure of the expression of the *S. mutans* gtfBC gene at pH 7.0 during transition periods between different growth rates. (A) Shift from a $0.05 \text{ h}^{-1}$ steady-state growth rate to $0.4 \text{ h}^{-1}$ at arrow; (B) shift from a $0.3 \text{ h}^{-1}$ steady-state growth rate to $0.05 \text{ h}^{-1}$ at arrow. Values are expressed as described in the legend to Fig. 2.

**FIG. 5.** CAT and extracellular GTF specific activities as a measure of the expression of the *S. mutans* gtfBC gene at pH 6.0 during transition periods between different growth rates. (A) Shift from a $0.05 \text{ h}^{-1}$ steady-state growth rate to $0.4 \text{ h}^{-1}$ at arrow; (B) shift from a $0.3 \text{ h}^{-1}$ steady-state growth rate to $0.05 \text{ h}^{-1}$ at arrow. Values are expressed as described in the legend to Fig. 2.
were lost the of BHI grated plasmid harboring of the markers in plating, respectively, medium for the duration of the experiments. Following the completion of a chemostat run, organisms were recovered from the vessel, briefly sonicated, and plated on BHI and BHI plus erythromycin Em to ascertain whether the fusions were lost at an appreciable frequency from the chromosomes of the test strains. Lower recoveries of organisms on erythromycin-containing medium would reflect loss of the integrated plasmid harboring the fusion and proximal promoter. There were no differences between the number of CFU of organisms on BHI and on BHI plus erythromycin (data not shown), suggesting that the marker was not lost at a appreciable frequency. The retention of CAT and erythromycin markers in the test strains as determined by assay and plating, respectively, was also considered to be indicative of stable maintenance of the fusion. Most notably, CAT expression levels from cells obtained from the final samplings of each experiment were comparable to those observed at the first steady-state time point and to those recorded by Hudson and Curtiss (24) in batch culture. Had a large proportion of the cells lost the fusion during the course of the experiments, then a significant decrease in total CAT specific activity would be predicted. This was never observed.

DISCUSSION

Microorganisms in the human mouth are subjected to radical and periodic changes in their environment. Because of intermittent eating patterns, vast differences in saliva quantity and composition associated with diurnal rhythms, mechanical forces, and specific and nonspecific host defenses, bacteria in plaque are continuously forced to adapt rapidly and efficiently to constantly changing conditions. S. mutans can persistently colonize a human host and, in certain instances, initiate dental caries (17) despite these environmental insults. The ability of S. mutans to modulate the expression of a variety of virulence determinants, including glucan and fructan metabolic machinery (26, 56), sugar transport systems (19), adhesins (42, 45), and aciduricity (2, 20), must be essential for the survival of this bacterium in the oral cavity. In order to understand how this organism persists and initiates disease and to devise novel and effective strategies to interfere with the disease process, it will be necessary to describe the phenotypic capabilities of this bacterium and to understand the molecular strategies underlying differential expression of its virulence factors.

For a long time, it was felt that the expression of the GTFs and FTF of S. mutans was constitutive and was not governed by complex regulatory circuits. However, studies performed by Walker et al. (56), Keevil et al. (28), and Jacques et al. (26) have clearly demonstrated that the production of exoenzymes of oral streptococci participating in the synthesis or breakdown of complex polysaccharides is markedly influenced by environmental conditions. These studies examined total glucan synthesis or structure but did not quantify individual enzymes. The data derived from examining steady-state cultures for GTF and FTF enzyme activity and CAT-gtf and -ftf specific activities indicate several key points regarding the regulatory signals controlling the expression of the gtf and ftf genes.

Environmental pH and the specific growth rate of the organisms dramatically influence transcriptional initiation from the ftf promoter and fructan synthesis capabilities. However, the influence of growth rate on ftf transcription occurs in a pH-dependent fashion. At pH 6.0, growth rate appears to have little influence on CAT-ff and CAT expression of extracellular FTF activity. On the other hand, the growth rate at pH 7.0 influences transcriptional activity and the amount of enzyme activity which can be determined in culture supernates. In this case, FTF activity and CAT-ff did not correlate well. In large part, this was due to the presence of greater amounts of total extracellular protein in faster-growth-rate cultures (data not shown), which resulted in an apparent decrease in FTF specific activity. However, posttranscriptional or posttranslational events may also control phenotypic expression of extracellular FTF, such as the release of cell-bound FTF (25). Alternatively, increases in expression of fructan hydrolase at pH 7.0 and $D = 0.3$ h$^{-1}$ may have resulted in the degradation of product fructans, although, in our experience, fructanase appears to have very little influence on FTF activity determined in vitro (57). The influence of pH and growth rate on ftf gene transcription is clear, but a more detailed study will be necessary to ascertain the molecular basis for the posttranscriptional control of FTF expression.
Under steady-state conditions, transcriptional activity from \textit{gfBC-cat} or total glucan synthesis is modestly influenced by the culture pH values utilized in this study or the growth rate of the organisms. Decreases in CAT-\textit{gf} activity are associated with increased growth rate. Although enzyme activity changes mimic changes in CAT-\textit{gf}, decreases associated with increased growth rate are not reflected in concomitant decreases of an identical magnitude in total GTF activity. This suggests that GTF-S activity (\textit{gfD}) may be regulated in a manner opposite that of \textit{gfBC}. Alternatively, dextranase may be regulated in a manner consistent with expression of \textit{GtfB} and \textit{GtfC}, and thus decreased product breakdown in reactions with supernatants from higher growth rates would be consistent. A final possibility, which will require much more extensive investigation is the control of expression of the \textit{GtfC} or \textit{GtfB} proteins by posttranscriptional events, particularly at the translational level (22).

Changes in the nutrient feed rate appear to elicit major changes in transcriptional activity and extracellular enzyme activity. In the oral cavity, the availability of nutrients to dental plaque varies considerably as a result of dietary intake of foodstuffs. For example, glucose concentrations in plaque can quickly change from 10 µM to 10 mM with ingestion of sweetened meals (8). Clearly, \textit{S. mutans} has evolved mechanisms to adapt to these and other environmental challenges. By increasing and decreasing the nutrient feed rate in the chemostat, organisms are faced with new growth parameters and are forced to respond to these changes by altering their physiology through modulation of gene expression patterns and allosteric control of enzyme reactions (10). The data derived from experiments examining transition-state expression of GTF and FTF plainly demonstrate that \textit{S. mutans} is capable of responding rapidly to these new conditions by altering transcription from both the \textit{gfBC} and \textit{ff} promoters. Moreover, the responses to the shift, as under steady-state conditions, occurred in a pH-dependent fashion, which may allow for differential expression of these gene products under ecologically disparate situations, e.g., those faced as an early colonizer of the tooth surface versus those which occur in mature plaque composed of a strongly acidogenic-aciduric microflora. These data strongly support that there is a hierarchy of control for expression of FTF and GTF, probably involving multiple variables. Another important point is that the levels of enzyme activity return to the respective steady-state levels relatively quickly, usually within 2 h. This observation indicates that regulation of expression, and not selection of subpopulations within the chemostat which may be better suited to growth under specific conditions, is responsible for the differences noted in CAT and extracellular enzyme activity.

It should be noted that transition states, as affected by alteration in the dilution rate of the chemostat vessel, do not simply reflect changing the growth rate of the organisms. With a change in the growth rate, there are changes in the limiting nutrient (i.e., glucose limited to glucose excess), changes in pH, ion concentration, and concentration of available amino acids, vitamins, and other nutrients. Thus, the signals received by the cell are likely complex and involve overlapping regions. In our experiments, a shift of the cells to a high growth rate resulted in a glucose-excess condition in the vessel during the transition, which is known to influence levels of phosphorylated components of the PTS system (52), activity and levels of glycolytic enzymes (7), and other regulatory circuits which could directly or indirectly affect GTF and FTF transcription, translation, and secretion. The use of transcriptional fusions in conjunction with mutants, such as \textit{Ei}^{high}-defective \textit{S. mutans} (21), with strains carrying mutations in the genes encoding the putative DNA-binding proteins associated with the \textit{ff} gene (44), or the use of strains with targeted mutations in the promoter regions of \textit{ff} or \textit{gfBC} should facilitate definition of the cis- and trans-acting regulatory components governing the differential expression of these genes in \textit{S. mutans} in response to environmental stimuli.

Recent experimentation with batch-grown \textit{S. mutans} cells has indicated that the synthesis of GTFs and FTF is regulated by the addition of sucrose, and twofold increases in enzyme synthesis have been recorded (24). Our data with the \textit{gfBC-cat} strain indicate that there is at least a threefold increase in activity from the \textit{gfBC} promoter when sucrose is added to slowly growing glucose-limited \textit{S. mutans} cells. This observation may be significant in terms of the response of \textit{S. mutans} to sucrose in dental plaque and may reflect mechanisms which have evolved in this bacterium to adapt quickly to the addition of sucrose in order to synthesize large quantities of water-insoluble glucans.

The fact that the large increase in CAT-\textit{gf} was not reflected in a similar magnitude of increase in the amount of glucan synthesized by culture supernates of cells induced by sucrose may be a result of multiple variables affecting the amounts of glucans synthesized during in vitro assays. One explanation for the disparity between CAT-\textit{gf} and GTF specific activities may be that in this study, only extracellular enzyme was assayed. It is known that when sucrose is present in the culture medium, a large increase in the proportion of GTF is found in a cell-associated form, presumably as a result of a complex of glucan-binding proteins, glucans, and GTF developing at the cell surface (17, 41, 50). Alternatively, the proportionately smaller increase in GTF specific activity to CAT-\textit{gf} may reflect down-regulation of the \textit{gfD} gene or other posttranscriptional events controlling GTF secretion or activity, which could result in a lower net yield of glucans. The attack of product glucans at α(1,6) linkages by endodextranase (54) may also have reduced the amount of glucan measured as precipitable material.

The data obtained in this study indicate that the operon fusions were stably maintained throughout the course of the experiments. The fusions were constructed by juxtaposing a fragment containing the 5' region of the \textit{gfBC} gene or \textit{ff} genes to a promoterless \textit{cat} gene and integrating this construct by an insertion-duplication mechanism into the \textit{S. mutans} UA130 chromosome. Because of the mechanism by which the foreign DNA was inserted, it was possible that the fusion could be excised from the chromosome and be lost from the strain by segregation. The inclusion of erythromycin in the culture medium provided selective pressure to maintain the construction in \textit{S. mutans}. Although the presence of erythromycin in the medium might have a minor effect on cell physiology, the experiments were confined to internal comparison of single strains under differing conditions. It is also noteworthy that the presence of an erythromycin resistance determinant in the construction did not alter the regulatory properties of the construct, nor does it seem to affect the ability of strains to colonize and elicit caries (23, 46, 57). Therefore, the use of erythromycin does not seem to be a disadvantage. Perhaps more importantly, insertion by Campbell model recombination, as was done for the strains utilized in this study, allows for constructing strains without insertionally inactivating the parent gene. During the course of these experiments, the fusion
strains likely had the duplicated promoter element in unit or near unit copy per chromosomal equivalent. Therefore, it is unlikely that the additional promoter element(s) had an impact on gene expression through titration of positive or negative trans-acting regulatory elements. The proximity of the CAT fusions to the cognate gtfBC and ftf promoters may have had some influence on transcription from these genes which was not readily apparent. Unfortunately, the alternative to these experiments would be to construct merodiploids in a recombination-deficient background or to integrate the fusion by double-crossover recombination, thereby inactivating the parent gene. For the former, such strains would potentially have inherent problems arising from titration of trans-acting regulatory elements, effects on cell physiology from harboring plasmid DNA, and physiologic defects arising from deficiency in the RecA-like enzyme of S. mutans, all of which could render these strains less fit than the wild type. For the second alternative, insertion by a double-crossover mechanism could inactivate the enzyme being studied or create polar effects which would prohibit assaying the gene product or otherwise confound the data. Therefore, the use of insertion-duplication seems a reasonable approach to establishing operon or gene fusions in strains of S. mutans.

In vivo, S. mutans is subjected to wide variations in sugar concentration and pH, and it will be of interest to study the effects of lower pH values and alternative carbon sources or concentrations on gene expression. Clearly, the gene fusion-chemostat technique presented here is applicable to studies examining a wide variety of culture conditions. Notwithstanding, the principal purposes of this investigation were to determine (i) whether gene fusions could be utilized stably in continuous culture to study transcriptional regulation of gene expression in S. mutans and potentially in other oral streptococci, (ii) whether transcription of GTFs and FTF was regulated by pH and/or growth rate, and (iii) whether GTF and FTF synthesis was inducible by sucrose in cells which were C limited and growing slowly, as in dental plaque. The pH values and growth rates chosen here were selected on the basis that pH 6.0 and 7.0 are physiologically relevant values for the oral streptococci. Also, previous chemostat studies have successfully demonstrated phenotypic changes in S. mutans in response to environmental stimuli, simulating similar culture conditions. This study demonstrated the utility of the technology and the influence of pH, growth rate, and sucrose on GTF and FTF expression and can now be expanded to experiments designed to study the influence of extreme pH values, carbon sources, and antimicrobial agents or other compounds on gene expression. It also seems feasible to use mixed cultures of oral bacteria to ascertain what influence co-culture of organisms has on gene expression.

Reporter gene fusions in conjunction with continuous culture represent a novel approach in the investigation of gene regulation in the oral streptococci. The data presented here demonstrate that many of the observed phenotypic changes result from differential control of transcription of GTF and FTF and the posttranslational events are key control points in expression of GTF and FTF activities. Since the ability of S. mutans to modulate expression of virulence determinants under various environmental conditions is likely crucial to the organism’s survival in the oral cavity, the dissection of regulation of individual genes in response to conditions commonly encountered in the mouth will be essential if the pathogenic strategies of this organism are to be understood. Likewise, such studies should provide insight into general molecular mechanisms governing pH, growth rate, and carbon source control of gene expression in prokaryotes. Moreover, this technique will be broadly applicable to a variety of virulence-related genes in oral streptococci and other oral pathogens. In combination with biochemical and immunologic techniques and experiments examining mRNA levels, analysis of the regulation of transcription of virulence determinants of oral bacteria should result in a more complete picture of the strategies utilized by oral pathogens to persist, to emerge within a plaque community, and to initiate disease.

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