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Differential expression profiles of *Streptococcus mutans ftf*, *gtf* and *vicR* genes in the presence of dietary carbohydrates at early and late exponential growth phases

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Abstract—Dental caries is one of the most common infectious diseases that affects humans. *Streptococcus mutans*, the main pathogenic bacterium associated with dental caries, produces a number of extracellular sucrose-metabolizing enzymes, such as glucosyltransferases (GTFB, GTFC and GTFD) and fructosyltransferase (FTF). The cooperative action of these enzymes is essential for sucrose-dependent cellular adhesion and biofilm formation. A global response regulator (*vicR*) plays important roles in *S. mutans ftf* and *gtf* expression in response to a variety of stimuli. A real-time reverse-transcription polymerase chain-reaction was used to quantify the relative levels of *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* transcription of *S. mutans* in the presence of various dietary carbohydrates: sucrose, D-glucose, D-fructose, D-glucitol (D-sorbitol), D-mannitol and xylitol. *Ftf* was highly expressed at late exponential phase in the presence of sorbitol and mannitol. *GtfB* was highly expressed in the presence of all the above carbohydrates except for xylitol at early exponential growth phase and glucose and fructose at late exponential growth phase. Similar to *gtfB*, the expression of *gtfC* was also induced with the presence of all the tested carbohydrates except for xylitol at early growth and glucose and fructose at late exponential phase. In addition, no effect of mannitol on *gtfC* expression at early exponential phase was observed. *GtfD* was less influenced compared to the *gtfB* and *gtfC*, demonstrating enhanced expression especially in the presence of sorbitol, glucose, mannitol and xylitol at early exponential phase and mannitol at late exponential phase. *VicR* expression was induced only at the presence of xylitol at late exponential phase, and a decrease in expression was recorded at early exponential phase. Our findings show that dietary carbohydrates have a major influence on the transcription of *ftf*, *gtfB*, *gtfC* and *gtfD*, but less on *vicR*. Sorbitol and mannitol, which are considered as noncariogenic sugar substitutes, may indirectly affect caries by promoting biofilm formation via enhanced expression of *gtf*s and *ftf*. These results suggest regulatory circuits for exopolysaccharide gene expression in *S. mutans*.

Keywords: Ftf; *Gtf*; *VicR*; Carbohydrates; Real-time RT-PCR; *Streptococcus mutans*; Gene expression

1. Introduction

Streptococcus mutans is one of the most important pathogens involved in the development of dental caries in humans.[1,2] A cardinal virulence property of *S. mutans* is its ability to produce extracellular polysaccharides from dietary carbohydrates.[3–5] *S. mutans* produces three glucosyltransferases (GTFs), GTFB, GTFC and GTFD, which synthesize glucan polymers from sucrose.[5] Adhesive glucans mediate the attachment of bacteria to the tooth surface as well as to other bacteria. *S. mutans* also produces a fructosyltransferase (FTF),[6] which synthesizes fructan polymers from sucrose.[7,8] Fructans are not only considered to function primarily as extracellular storage compounds,[9] but can also act as binding sites for bacterial accumulation.[10]

The ability of *S. mutans* to colonize on teeth is paramount to the initiation and progression of dental caries.[2,11,12] A number of studies have indicated that the expression of genes encoding the exopolysaccharide-synthesizing enzymes of *S. mutans* is influenced by environmental pH, growth rate, carbon sources,[13–15] and is also genetically regulated.[16–18]

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Bacteria exposed to transient environments can elicit adaptive responses by triggering differential expression of genes via two regulatory elements consisting of a histidine kinase and response regulator. *VicRK* signal transduction system is involved in regulating several important physiological processes in *S. mutans*.[16,19] The *vic* gene products appear to regulate the expression of several virulence-associated genes affecting synthesis and adhesion to polysaccharides, including *ftf*, *gtfBCD* and *gbpB*.[16]

The bulk of the research on *S. mutans* gene expression[13,14,20] has been carried out using media containing glucose or sucrose as the primary carbon source. Since D-glucitol (sorbitol) is one of the most common artificial sweeteners found in 'sugar-free' products, it was of interest to investigate its effect on the expression of *ftf*, *gtf* and *vicR* of *S. mutans* and to compare it with that of cells growing in the presence of mannitol or xylitol.

Most *S. mutans* strains are distinguished from other oral bacteria by their ability to utilize sorbitol as the sole carbon source.[21] Sorbitol and mannitol have a limited effect on acid production by mutans streptococci in contrast to sucrose, fructose and glucose.[22] Although sorbitol is a hexitol, it has two more hydrogen atoms than glucose or fructose; therefore, it is less efficiently utilized by mutans streptococci, as its metabolism requires the induction of specific, energy-dependent transport systems and specific metabolic pathway.[23,24] In contrast to sorbitol, xylitol is a sugar substitute that is not metabolized by mutans streptococci.[22,25]

The purpose of this study was to differentiate and quantify the expression of *S. mutans ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* at early and late exponential growth phases in the presence of cariogenic and noncariogenic carbohydrates using real-time quantitative PCR.

2. Results and discussion

In this study, we analyzed the effect of several common dietary simple sugars on the expression of genes associated with biofilm formation. A different pattern of expression of those genes in the planktonic state is a pivotal step in the initiation of adhesion and biofilm formation. Therefore, it is of importance to examine the effect of carbohydrates on the expression of those genes prior to the adhesion stage.

Equal amounts of total RNA (1 μg) from early-exponential and late-exponential phase cultures were used to quantify the transcript levels of *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR*. All isolated RNA samples contained negligible amounts of double-stranded DNA. Using the designed primer sets, we differentiated these genes by real-time RT-PCR using SYBR Green, and the specificity of the PCR products was confirmed by dissociation curve analysis.

Our findings show that mRNA expression of *ftf*, *gtfB*, *gtfC* and *gtfD* by *S. mutans* GS5 is carbohydrate-regulated; sucrose, fructose and glucose enhanced *ftf*, *gtfB* and *gtfC* expression (Figs. 1–3). In addition, we found that such expression was more pronounced in the early bacterial exponential phase, compared with the late exponential phase, indicating that regulation of these genes by carbohydrates is more likely when bacteria are in the stage of intense protein synthesis. *GtfD* expression was not significantly affected by sucrose, fructose or glucose (Fig. 4).

Sorbitol and mannitol are considered noncariogenic carbohydrates and are often used as substitutes for sucrose, glucose or fructose. A notable finding in this study is the effect of these noncariogenic carbohydrates

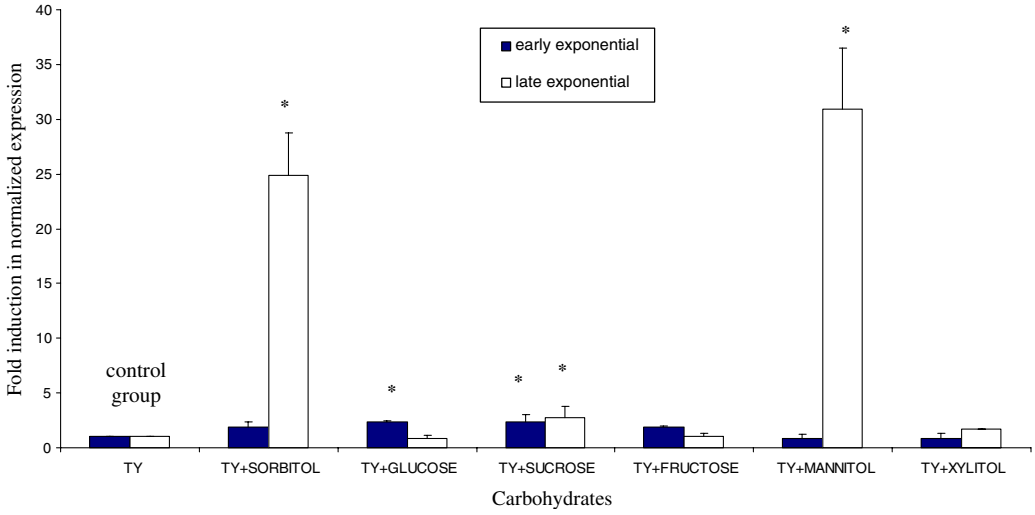

Figure 1. Effects of sucrose, glucose, fructose, sorbitol, mannitol and xylitol on *ftf* expression in early- and late-exponential growth phase of *S. mutans* GS5 grown in TY medium. The mRNA expression levels were calibrated relative to the control group in early and late growth phases. The results are expressed as the means and standard deviations of triplicate experiments using primers specific for *ftf* and 16S rRNA (normalizing gene). *Statistical differences ($p < 0.05$) between gene expressions in the presence of various carbohydrates supplemented in TY and control group of TY without carbohydrates.

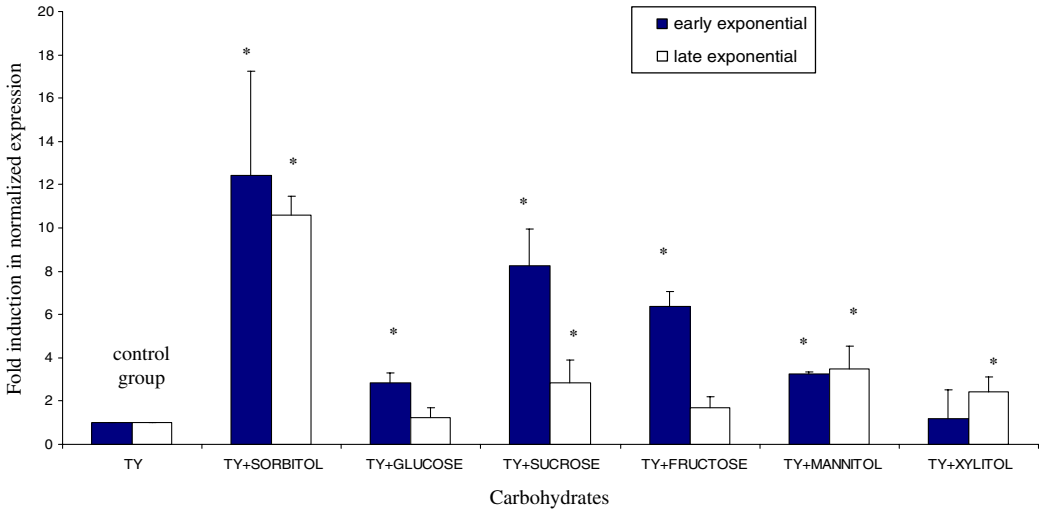

Figure 2. Effects of carbohydrates on *S. mutans gtfB* expression in early- and late-exponential growth phase. The mRNA levels were calibrated according to the control group in early- and late-exponential growth phase. The data are expressed as the means and standard deviations of duplicate experiments. *Differences ($p < 0.05$) between gene expressions in the presence of various carbohydrates supplemented in TY and control group of TY without carbohydrates.

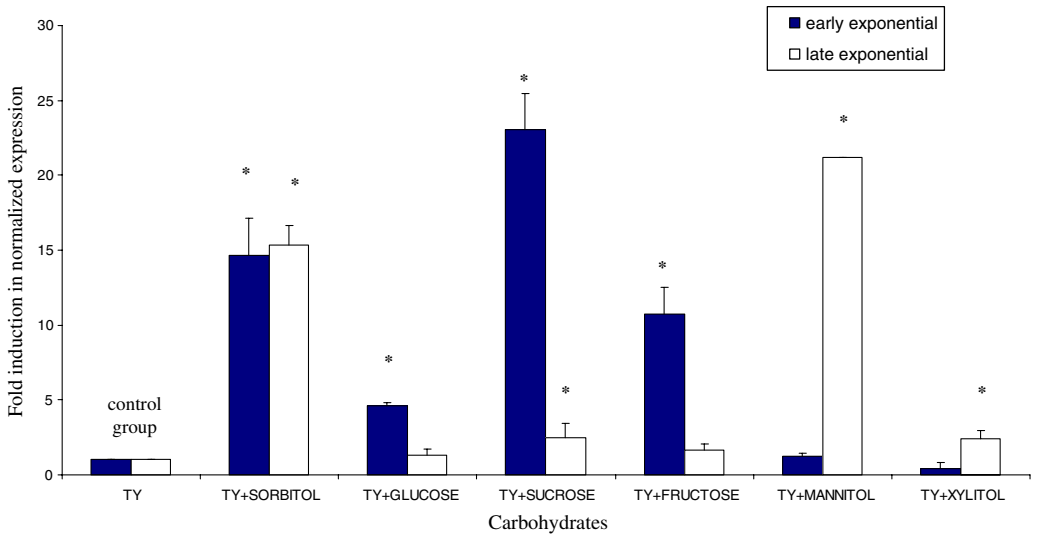

Figure 3. Effects of carbohydrates on *S. mutans gtfC* expression in early- and late-exponential growth phase. The mRNA levels were calibrated according to the control group in early- and late-exponential growth phase. The data are expressed as the means and standard deviations of duplicate experiments. *Differences ($p < 0.05$) between gene expressions in the presence of various carbohydrates supplemented in TY and control group of TY without carbohydrates.

on the expression patterns of *ftf*, *gtfB*, *gtfC* and *gtfD*. The most significant increase in *ftf* expression was observed in the presence of sorbitol and mannitol in the late exponential phase (25-fold and 31-fold, respectively, compared with the control), while the other carbohydrates evaluated had a minor effect on *ftf* expression in early or late exponential phase (Fig. 1). The expression profile of *gtfD* was affected mainly by sorbitol and mannitol in the late exponential phase like *ftf* expression (Fig. 4). Sorbitol also enhanced the expression of *gtfB* and *gtfC* in both early and late exponential phases (Figs. 2 and 3). Sorbitol, one of the most common sugar substitutes, is utilized via the phosphotransferase system of *S. mutans* only after an adaptation period in which the sorbitol is converted into phosphorylated fructose.[25–27] The consequent reduction in pH values after utilization of sorbitol is less than that after metabolism of glucose, fructose or sucrose.[21] In addition, since *S. mutans* does not utilize sorbitol as a source for glucan or fructan synthesis,[23,26] it was assumed that sorbitol does not play a significant role in sucrose-dependent adhesion and dental biofilm formation.[23,26,27] However, our study shows that the expression of *ftf*, *gtfB* and *gtfC* genes is significantly induced by the presence of sorbitol. This may

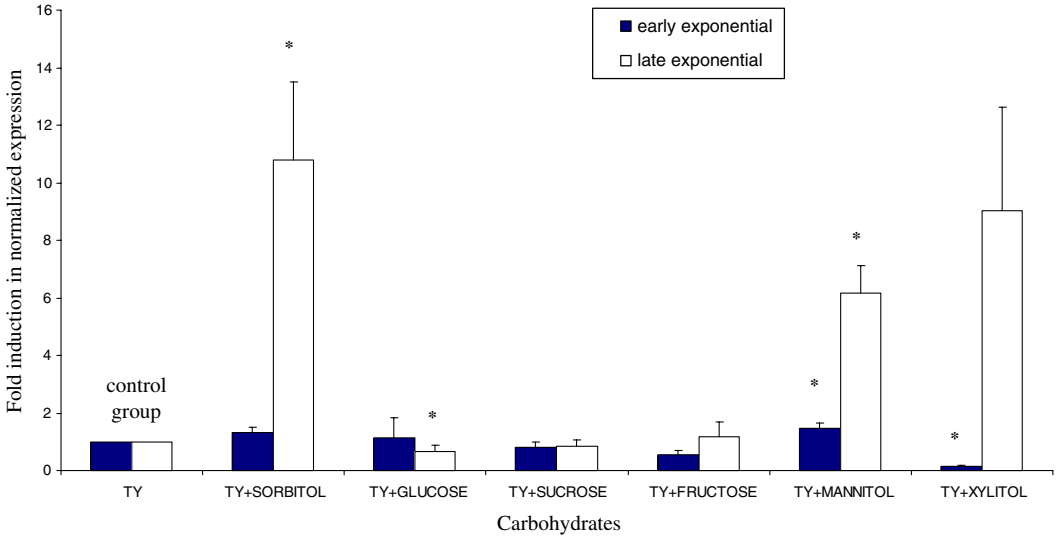

Figure 4. Effects of carbohydrates on *S. mutans gtfD* mRNA expression in early- and late-exponential growth phase. The mRNA levels were calibrated according to the control group in early- and late-exponential growth phase. The data are expressed as the means and standard deviations of duplicate experiments. *Differences ($p < 0.05$) between gene expressions in the presence of various carbohydrates supplemented in TY and control group of TY without carbohydrates.

indicate that sorbitol indirectly affects biofilm formation by enhancing the synthesis of GTF and FTF enzymes, both involved in biofilm formation and bacterial accumulation when sucrose is present.

Xylitol, a nonfermentable artificial sweetener, is also used as a noncariogenic sugar substitute similar to the use of sorbitol. It has been reported lately that xylitol enhances *gbp* expression,[28] a gene-encoding, glucan-binding protein located on the membrane of oral bacteria that plays a crucial role in bacterial adhesion.[29,30] This information indicates that although xylitol does not induce pH reduction nor serves as a substrate for GTF or FTF activity, it might play a role in biofilm formation by enhancing *gbp* expression. According to our results, xylitol did not significantly effect *ftf*, *gtfB* and *gtfC* expression. However, *gtfD* and *vicR* transcription levels were sharply decreased in the early exponential phase and were significantly enhanced in the late exponential phase in the presence of xylitol (Figs. 4 and 5).

Sucrose, fructose and glucose enhanced *gtfB* and *gtfC* expression in the early exponential phase to a greater extent than in the late exponential phase (Figs. 2, 3). In the presence of sucrose, increased expression of *ftf*

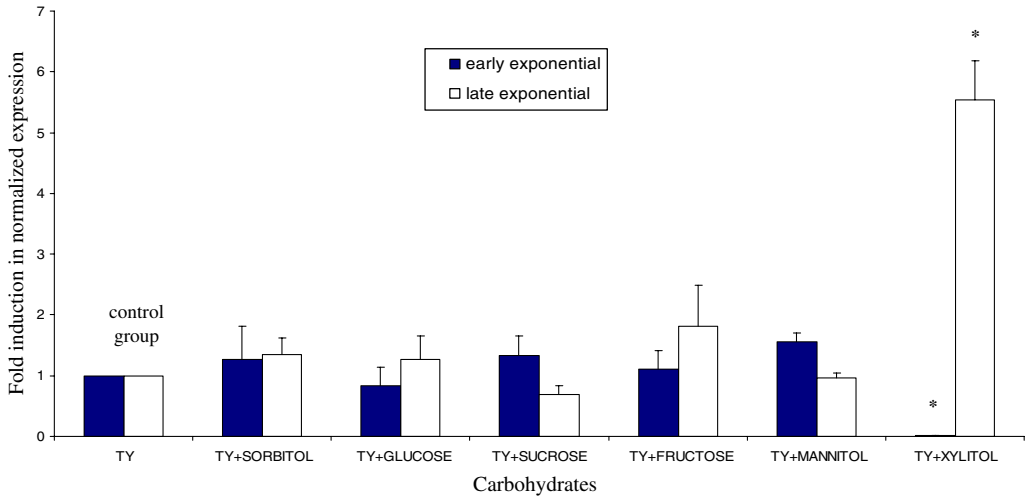

Figure 5. Effects of carbohydrates on *vicR* mRNA expression in early- and late-exponential growth phase of *S. mutans*. The mRNA levels were calibrated according to the control group in early- and late-exponential growth phase. The data are expressed as the means and standard deviations of duplicate experiments. *Differences ($p < 0.05$) between gene expressions in the presence of various carbohydrates supplemented in TY and control group of TY without carbohydrates.

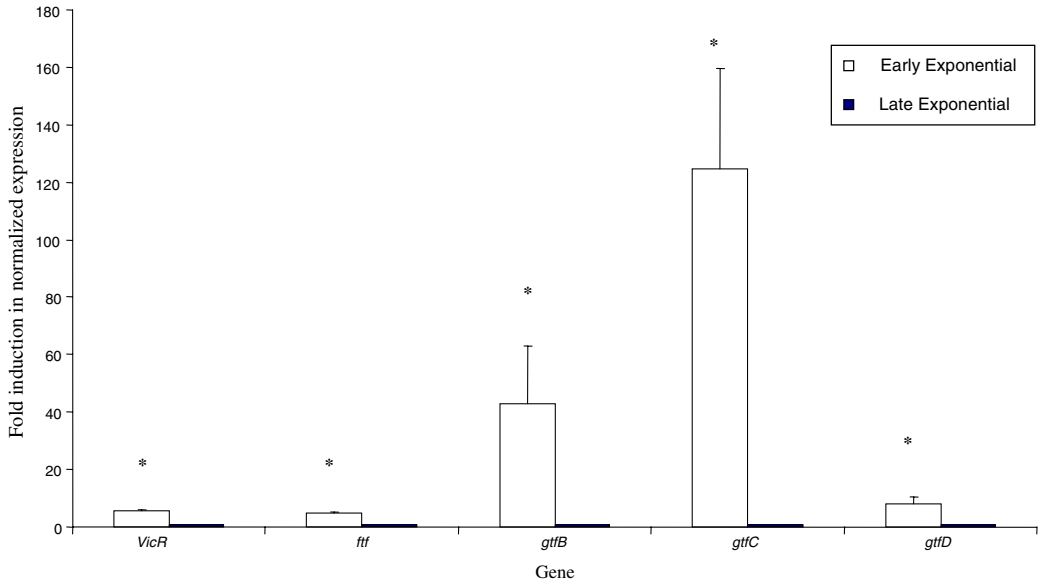

Figure 6. Relative quantities of *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* mRNA expression in early- and late-exponential growth phase of *S. mutans* GS5 grown in TY medium. The mRNA levels were calibrated according to the expression in late phase growth. The data are expressed as the means and standard deviations of duplicate experiments. *Differences ($p < 0.05$) between gene expressions in early- and late-exponential growth phase.

(Fig. 1), and especially *gtfB* and *gtfC* mRNA (Figs. 2 and 3), was noted. Sucrose increased the *gtfC* expression by 23-fold in early exponential phase; its effect in late exponential phase was minimal (Fig. 3).

Dramatic transcriptional and physiological changes accompany growth in early and late exponential growth phase in a planktonic environment.[31,32] It is of interest that the effects of growth phase on the expression patterns of the tested genes were different. The level of mRNA for all of the differentiated genes was significantly higher in early than in the late exponential phase (Fig. 6). The expression of *gtfB* and *gtfC* was sharply upregulated by 43- and 124-folds, respectively. However, the level of *ftf*, *gtfD* and *vicR* genes mRNA was increased much more moderately between the early- and late-exponential phase. These results confirm previous reports that have noted that *gtf* genes are independently expressed.[33,34]

Previous studies on *S. mutans* MT8148, using real-time RT-PCR, have shown decreased expression of *gtfB* and *gtfC*, although twice the amount of *gtfD* mRNA was detected in the presence of 2% sucrose.[20] A plasmid-based reporter system using a luciferase assay has revealed that the expression of the upstream regions of *gtfB*/*C* remained constant in the presence of sucrose, glucose, and fructose.[35] On the other hand, using the promoterless chloramphenicol acetyltransferase (CAT) gene integrated into the chromosomal *gtfB* gene, investigators reported that the expression of the *S. mutans* *gtfB*/*C* operon was stimulated in the presence of sucrose.[14] Other studies showed that sucrose induced the expression of *ftf*-CAT and *gtfB*/*C*-CAT in *S. mutans*.[15,36] Sucrose also induced *ftf*-CAT expression in *S. mutans* present on teeth in a mammalian host (rat).[37] The addition of glucose or fructose to *S. mutans* biofilms did not induce *ftf*-CAT expression.[15,36] The above conflicting results from the literature indicate that the expression of those genes is dependent on several factors such as bacteria strains, growth phase, and other environmental parameters as growth media and carbohydrate content. Clearly further investigations are required for understanding the exact mechanism involved in the regulation of *ftf* and *gtf* gene expression by the various strains of mutans streptococci as one means to prevent biofilm formation.

In conclusion, differential analysis of *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* transcripts from *S. mutans* grown in the presence of carbohydrates at early and late exponential growth phases revealed significant shifts in the expression of these genes involved in biofilm formation. Our data indicate that sorbitol and mannitol may indirectly affect the caries process, as it upregulates the expression of genes encoding enzymes that are associated with biofilm formation.

3. Experimental

3.1. Tested carbohydrates

D-Glucose (E. Merck, Darmstadt, Germany); D-fructose (E. Merck); and D-glucitol (BDH Laboratory, England); sucrose (Frutarom Ltd, Israel); D-mannitol (E. Merck); xylitol (Sigma–Aldrich, St. Louis, MO, USA).

3.2. Bacterial strains and culture conditions

S. mutans GS5, a human clinical isolate commonly used in dental research, was grown overnight at 37 °C in TY medium (1.4% tryptone and 0.8% yeast extract) in an atmosphere of 5% CO_2. To analyze the effect of simple sugars on the gene expression, the organism was cultured in TY medium supplemented with 10 g/L sucrose, 10 g/L glucose, 10 g/L fructose, 10 g/L sorbitol, 10 g/L mannitol or 10 g/L xylitol as the carbohydrate source.

3.3. Extraction of total RNA

Cultures of *S. mutans*, grown as described above, were diluted at a ratio of 1:50, inoculated into fresh TY media and grown to early exponential phase (culture $OD_{590} = 0.2$, pH 6.5 ± 0.2), or late-exponential phase (culture $OD_{590} = 0.5$, pH 5.5 ± 0.25). The cells were collected by centrifugation (5000*g* at 4 °C for 5 min) and then resuspended in Tri-Reagent (Sigma–Aldrich, St. Louis, MO, USA). The cells were disrupted with the aid of a Fast Prep Cell Disrupter (Bio 101, Savant Instruments, Inc., NY, USA) and centrifuged, and the RNA containing supernatant was supplemented with 1-bromo-3-chloropropane (BCP) (Molecular Research Center, Cincinnati, OH, USA). The upper aqueous phase was precipitated with 2-propanol. After centrifugation, the resulting RNA pellet was washed with 75% ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Because of the sensitivity of PCR, residual contaminating DNA was eliminated by incubation of the sample with RNase-free DNase (Promega, Madison, WI, USA). The DNase was then inactivated by incubation at 65 °C for 10 min, and the RNA was precipitated with ethanol and suspended in diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was determined spectrophotometrically using the Nanodrop Instrument (ND-1000, Nanodrop Technologies, Wilmington, DE, USA). The integrity of the RNA was assessed by agarose gel electrophoresis (data not shown).

3.4. Reverse transcription

A reverse transcription (RT) reaction mixture (20 μL) containing 50 ng of random hexamers, 10 mM dNTPs mix and 1 μg of total RNA sample was incubated at 65 °C for 5 min to remove any secondary structure, and placed on ice. Then $10 \times$ RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U of RNaseOUT Recombinant Ribonuclease Inhibitor and 50 U of Super Script II RT (Invitrogen, Life Technologies, Carlsbad, California, USA) were added to each reaction mix. After incubation at 25 °C for 10 min, the mix was incubated at 42 °C for 50 min. Heating the mixture at 70 °C for 15 min terminated the reaction, and the cDNA samples were stored at −20 °C until used.

3.5. Real-time quantitative PCR

Amplification, detection, and analysis of mRNA were performed using the ABI-Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with an SYBR Green PCR Master Mix (Applied Biosystems). The *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* primers were designed using the algorithms provided by Primer Express (Applied Biosystems) for uniformity in size (≈95 bp) and melting temperature. For each set of primers, a standard amplification curve (critical threshold cycle against exponential of concentration) was plotted, and only those with slope ≈ −3 were considered reliable primers (Supplementary data, Figs. S1–6). The primer sequences are provided in Table 1.

The reaction mixture (20 μL) contained $1 \times$ SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of the cDNA sample, and 0.5 μM of the appropriate forward and reverse PCR primers. PCR conditions included an initial denaturation at 95 °C for 10 min, followed by a 40-cycle amplification consisting of denaturation at 95 °C for 15 s and annealing and extension at 60 for 1 min. All primer pairs were checked for primer–dimer formation by using the two-step protocol

Table 1. Nucleotide sequences of primers for genes whose expression at early and late exponential growth phases was compared in the presence of carbohydrates

Primer	Sequence (5′–3′)	Fragment location	Accession number
Ftf-F	AAATATGAAGGCGGCTACAACG	1358–1379	M18954
Ftf-R	CTTCACCAGTCTTAGCATCCTGAA	1435–1458	M18954
GtfB-F	AGCAATGCAGCCAATCTACAAAT	1150–1172	M17361
GtfB-R	ACGAACTTTGCCGTTATTGTCA	1224–1245	M17361
GtfC-F	CTCAACCAACCGCCACTGTT	434–453	M22054
GtfC-R	GGTTTAACGTCAAAATTAGCTGTATTAGC	496–524	M22054
GtfD-F	ACAGCAGACAGCAGCCAAGA	1365–1384	M29296
GtfD-R	ACTGGGTTTGCTGCGTTTG	1440–1458	M29296
VicR-F	TGACACGATTACAGCCTTTGATG	184–206	AF393849
VicR-R	CGTCTAGTTCTGGTAACATTAAGTCCAATA	255–284	AF393849
16S-F	CCTACGGGAGGCAGCAGTAG	243–262	X58303
16S-R	CAACAGAGCTTTACGATCCGAAA	321–343	X58303

described above without the addition of RNA template. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out in the absence of reverse transcriptase in order to identify whether residual genomic DNA contaminated the RNA samples. The critical threshold cycle (C_t) was defined as the cycle in which fluorescence becomes detectable above the background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with C_t values obtained from the amplification of known quantities of *S. mutans* cDNA. The standard curves were used for transformation of the C_t values to the relative number of cDNA molecules. The contamination of genomic DNA was determined from control reactions, devoid of reverse transcriptase. The same procedure was repeated for all the primers.

The expression levels of all the tested genes (*ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR*) were normalized using the 16S rRNA gene of *S. mutans* (Acc. No. X58303) as an internal standard. There was no significant difference in the expression of the 16S rRNA gene in the various tested conditions and samples (Supplementary data, Fig. S7). Each assay was performed with at least two independent RNA samples in duplicate, and the *x*-fold change of the transcription level was calculated by the following equations (ABI Prism 7000 SDS Software v1.1 with RQ Study 1.0, Applied Biosystems):

$$\text{Each cDNA: } \Delta C_t = C_{t\,(\text{target gene})} - C_{t\,(16S\ \text{rRNA})}, \quad (1)$$

$$\Delta\Delta C_t = \Delta C_{t\,(\text{reference cDNA})} - \Delta C_{t\,(\text{test cDNA})}, \quad (2)$$

$$\text{Ratio} = 2^{-\Delta\Delta C_t}. \quad (3)$$

3.6. Statistical analysis

One tail Student's *t*-test was used to calculate the significance of the difference between the mean expression of a given experimental samples and the control samples. A p value of <0.05 was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.05.010.

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