***Streptococcus mutans* biofilm formation: utilization of a *gtfB* promoter–green fluorescent protein (*PgtfB::gfp*) construct to monitor development**

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The glucosyltransferases of *Streptococcus mutans* are recognized as important virulence factors for this cariogenic bacterium. To study the expression of the *gtfB* gene of *S. mutans* in biofilms, a *gtfB* promoter (*PgtfB*)–green fluorescent protein (GFP) reporter system was developed. A *Streptococcus–Escherichia coli* shuttle vector harbouring a *PgtfB::gfp* cassette was introduced into *S. mutans* GS-5, and the expression of GFP by the transformed *S. mutans* cells was confirmed by fluorescence microscopy. Furthermore, confocal laser scanning microscopy was carried out on biofilms attached to polystyrene plates; enhanced *gtfB* expression was observed in various microcolonies across these biofilms. To further test the hypothesis that *gtfB* expression is upregulated in biofilms, flow cytometry analysis was done on planktonic and biofilm cells; this analysis showed an approximately five-fold increase in *gtfB* expression in the biofilm cells relative to the planktonic cells. Real-time (TaqMan) PCR analysis confirmed that *gtfB* expression in the biofilm cells was enhanced relative to the planktonic cells. Previously, it has been suggested that the *S. mutans gtfC* gene might be co-transcribed with *gtfB*. Therefore, RT-PCR analysis was performed on *gtfB*-expressing *S. mutans*; this analysis demonstrated that *gtfC* was co-transcribed with *gtfB*. These results indicated that GFP expression can be utilized to examine gene regulation in *S. mutans* biofilm formation.

Keyword: glucosyltransferase (GTF)

INTRODUCTION

The extracellular polysaccharides synthesized from sucrose via the glucosyltransferases (GTFs) of *Streptococcus mutans* have been recognized to be important virulence factors for the induction of human dental caries (Loesche, 1986). *S. mutans* synthesizes α -(1→3)- and α -(1→6)-linked glucan polymers through the concerted action of three secreted GTFs that are encoded by the genes *gtfB*, *gtfC* and *gtfD*. The tandemly arranged *gtfB* and *gtfC* genes encode the enzymes (GTF-I and GTF-SI, respectively) responsible for the synthesis of water-insoluble, α -(1→3)-rich glucan (IG) (Aoki *et al.*, 1986; Hanada & Kuramitsu, 1988). *gtfD* encodes GTF-

S, which is responsible for the synthesis of α -(1→6)-linked, water-soluble glucan (SG) (Hanada & Kuramitsu, 1989). The principal roles of the extracellular glucans of *S. mutans* are to facilitate the adhesion of the organism to and its accumulation on teeth, and to establish an extracellular polysaccharide matrix for the organism that may provide it with increased protection against mechanical host-clearance forces and which may afford it some protection against host immune defences. *S. mutans* cells attached to teeth as part of a biofilm (dental plaque) may exhibit a distinct mode of growth which differs from that of planktonic cells, and this distinct mode of growth can be characterized by an increased resistance to antibiotics as well as by differential gene expression (Watnick & Kolter, 2000).

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a visual marker of gene expression in prokaryotic and eukaryotic organisms. GFP has the advantage of being more sensitive

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Abbreviations: CDM, chemically defined medium; CLSM, confocal laser scanning microscopy; Ct, threshold cycle; *gfp* and GFP, green fluorescent protein; *gtf* and GTF, glucosyltransferase; *PgtfB*, *gtfB* promoter.

than other reporter genes and requires no extraneous substrates or co-factors for its detection (Chalfie *et al.*, 1994). Furthermore, mutants of GFP with increased fluorescence intensities have been constructed; for example, one GFP mutant, *gfp-mut2*, contains a triple substitution – S65A, V68L and S72A – and exhibits 100-fold more fluorescence in *Escherichia coli* than the wild-type protein (Cormack *et al.*, 1996). GFP has also been used as a species-specific marker to label adherent bacteria in biofilms (Skillman *et al.*, 1998). Furthermore, GFP can be used to analyse the spatial distribution of bacteria in biofilms. Oral biofilms, in the form of dental plaque, are particularly complex because they consist of hundreds of bacterial species (Davey & O'Toole, 2000); hence, the utilization of GFP to examine these communities should provide further insights into the spatial distribution of organisms within dental plaque.

Previously, molecular genetic and biochemical studies of the *gtf* genes have been carried out using planktonic *S. mutans*. *In vitro* studies have indicated that *gtfB* and *gtfC* are essential for the sucrose-dependent attachment of *S. mutans* cells to hard surfaces (Aoki *et al.*, 1986) but *gtfD* is dispensable (Hanada & Kuramitsu, 1989). Nevertheless, all three genes appear to be required for induction of maximal dental caries in a rat model (Yamashita *et al.*, 1993). In addition, by using a chloramphenicol reporter gene, it has been demonstrated that expression of the *gtfB-gtfC* operon of *S. mutans* appears to be induced when the organism colonizes solid surfaces *in vitro* (Hudson & Curtiss, 1990). However, since *gtfC* can be transcribed from its own promoter (Smorawinska & Kuramitsu, 1995), it is not clear if these effects were a result of increased transcription from *gtfB*, *gtfC* or both promoters. To test the hypothesis that the expression of *S. mutans gtfB* is upregulated in biofilms, we have developed a *Streptococcus-E. coli* shuttle vector system to quantitate and visualize the expression of this gene in biofilms using plasmid-encoded GFP. The results described here confirm that *gtfB* expression is upregulated in biofilm cells and also indicate that the transcription of *gtfC* can be initiated from the *gtfB* and the *gtfC* promoters.

METHODS

Bacterial strains and plasmids. All of the bacterial strains and plasmids used in this study are listed in Table 1. Strains of *S. mutans* were routinely subcultured and maintained in Todd-Hewitt broth (THB; Difco). Transformants of *S. mutans* were selected following their growth on THB agar plates supplemented with 10 µg erythromycin ml⁻¹ or 500 µg kanamycin ml⁻¹.

DNA manipulations. DNA isolation, restriction digests, ligations and transformations of competent *E. coli* cells were carried out as described by Sambrook *et al.* (1989). Transformation of *S. mutans* was performed as described previously (Perry *et al.*, 1983).

Construction of *PgtfB::gfp* fusion plasmids. The primers used in this study are listed in Table 2. Plasmid pKEN, containing *gfp-mut2*, was described by Cormack *et al.* (1996). The

promoterless *gfp XbaI-PstI* cartridge was excised from pKEN and inserted into the *XbaI-PstI* sites of pUC19 to generate pAYG210. For amplification of the *S. mutans* GS-5 *PgtfB* region, oligonucleotides were synthesized based upon the nucleotide sequence of the promoter region of *gtfB* including a portion of the 5' region of *gtfB* (GenBank accession no. M17361). To facilitate cloning of the PCR product into pAYG210, which contained the promoterless *gfp-mut2* sequence, restriction sites were incorporated into the primers. The primer pair comprised *gtfBF520*(Bam) (complementary to nucleotides 520–537) and *gtfBR712*(Xba) (complementary to nucleotides 695–712). A PCR product was amplified from *S. mutans* GS-5 chromosomal DNA. The product was digested with *Bam*HI and *Xba*I and inserted in-frame into similarly digested pAYG210. A *gtfB::gfp* fusion fragment, digested with *Bam*HI and *Pst*II, from pAYBG310 was amplified by PCR using the primer pair *gtfBF520*(Sph)/*gfpR*(Sph) (Prasher *et al.*, 1992). The resulting PCR product was digested with *Sph*I and inserted into *Sph*I-digested pDL276 (Dunny *et al.*, 1991); the resulting plasmid was designated pAYBG754S. To introduce erythromycin resistance into pAYBG754S, the erythromycin cassette was amplified by PCR from pResEmMCS10 using primers AM1(Sma) and AM3(Sma), respectively (Brehm *et al.*, 1987; Shiroza & Kuramitsu, 1993). The resulting PCR product was digested with *Sma*I and inserted into the *Sma*I site of pAYBG754S, to generate the erythromycin-resistant plasmid pAYBG854S. To generate a *gtfB::gfp* fusion by a Campbell-like insertion into the chromosome of *S. mutans*, a suicide plasmid harbouring the *gtfB::gfp* fusion was constructed. A *gtfB::gfp* fusion fragment, digested with *Bam*HI and *Hind*III, from pAYBG310 was inserted into the *Bam*HI- and *Hind*III-digested integration plasmid pZ63 (Sato *et al.*, 1991); the resulting plasmid was designated pAYBG554.

Fluorescence microscopy. Cultures of *S. mutans* strains were grown in chemically defined medium (CDM) supplemented with sucrose to an OD₅₇₀ value of 0.5. The CDM contained (l⁻¹) 2.0 g L-glutamic acid, 0.2 g L-cysteine, 0.9 g L-leucine, 1.0 g NH₄Cl, 2.5 g K₂HPO₄, 2.5 g KH₂PO₄, 4.0 g NaHCO₃, 1.2 g MgSO₄·7H₂O, 0.02 g MnCl₂·4H₂O, 0.02 g FeSO₄·7H₂O, 0.6 g sodium pyruvate, 1.0 mg riboflavin, 0.5 mg thiamin/HCl, 0.1 mg D-biotin, 1.0 mg nicotinic acid, 0.1 mg *p*-aminobenzoic acid, 0.5 mg calcium pantothenate, 1.0 mg pyridoxal/HCl and 0.1 mg folic acid, and was adjusted to pH 7.0 with H₃PO₄. To maximize *gfp* expression, 1.0 M phosphate buffer was added to the cultures to a final concentration of 50 mM (pH 7.0) about 10 min before the microscopic analysis was done. The cell cultures were analysed directly by using phase-contrast fluorescent microscopy.

Flow cytometry analysis. *gtfB* expression by cells of *S. mutans* 854S and GS-5 in their planktonic and biofilm forms was monitored by flow cytometry using an EPICS XL apparatus with XL SYSTEM II software (Beckman Coulter). Green fluorescence ($\lambda_{\text{excitation}}$ 488 nm; $\lambda_{\text{emission}}$ 515–545 nm) was analysed. Dead cells were excluded from the analysis using forward- and side-scatter light gating. Planktonic and sessile cells for fluorescence-activated cell sorter (FACS) analysis were obtained following overnight anaerobic culturing of the strains in CDM supplemented with 0.5% sucrose in 6-well polystyrene plates (Corning). Under these conditions, the bottom surfaces of the wells were uniformly covered by the parental GS-5 cells. Briefly, after separating the planktonic and sessile cells, the sessile cells were washed several times with CDM. The sessile cells were then resuspended in fresh CDM and sonicated to disrupt the streptococcal chains, to avoid the effects of size differences. The planktonic cells were also sonicated, in a similar manner to the sessile cells. Both

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Source/reference
<i>S. mutans</i> GS-5	Wild-type; Em ^s	SUNYaB†
<i>S. mutans</i> 554	GS-5 containing chromosomally integrated pAYBG554; Em ^r Km ^r	This study
<i>S. mutans</i> 854S	GS-5 containing pAYBG854S; Em ^r Km ^r	This study
<i>E. coli</i> DH5 α	Cloning host	Gibco-BRL
Plasmids		
pUC19	Cloning vector; Amp ^r	New England Biolabs
pKEN	<i>gfp-mut2</i> ; Amp ^r	Cormack <i>et al.</i> (1996)
pAYBG310	pUC19 harbouring <i>PgtfB::gfp</i> ; Amp ^r	This study
pDL276	<i>Streptococcus-E. coli</i> shuttle vector; Km ^r	Dunny <i>et al.</i> (1991)
pZ63	<i>Streptococcus</i> integration plasmid harbouring <i>scrB::lacZ</i> ; Em ^r Km ^r	Sato <i>et al.</i> (1991)
pAYBG554	pZ63 harbouring <i>PgtfB::gfp</i> instead of <i>scrB::lacZ</i> ; Em ^r Km ^r	This study
pAYBG754S	pDL276 harbouring <i>PgtfB::gfp</i> ; Km ^r	This study
pResEmMCS10	<i>Streptococcus</i> integration plasmid; Em ^r Amp ^r	Shiroza & Kuramitsu (1993)
pAYBG854S	pDL276 harbouring <i>PgtfB::gfp</i> ; Em ^r Km ^r	This study

* Em^s, erythromycin-sensitive; Em^r, erythromycin-resistant; Km^r, kanamycin-resistant; Amp^r, ampicillin-resistant.

† SUNYaB, Culture Collection of the Department of Oral Biology, State University of New York, Buffalo, NY, USA.

Table 2. Oligonucleotide primers and probes used in this study

Oligonucleotide	Sequence*	Gene(s)
Primer		
gtfBF520(Bam)	5'-CCC CAT CGA TAG ACT GTT GTT TGG TTG-3'	<i>PgtfB</i>
gtfBR712(Xba)	5'-CCC CTC TAG ACC ATT AGG AAC CTC CAA A-3'	<i>PgtfB</i>
gtfBF520(Sph)	5'-CCC CGC ATG CAG ACT GTT GTT TGG TTG-3'	<i>PgtfB::gfp</i>
gfpR(Sph)	5'-CCC CGC ATG CTT ATT TGT ATA GTT CAT C-3'	<i>PgtfB::gfp</i>
AM1(Sma)	5'-CCC CCC GGG GAA GGA GTG ATT ACA TGA AC-3'	Erythromycin cassette
AM3(Sma)	5'-CCC CCC GGG AGC GAC TCA TAG AAT TAT TTC-3'	Erythromycin cassette
RT1F	5'-TAC TTT AGA GCA AAC GGT-3'	<i>gtfBC</i>
RT1R	5'-AGC AGT CAG AGT TTC TTT-3'	<i>gtfBC</i>
RT2F	5'-CTT ATA CTG TTG GGG AAA-3'	<i>gtfB</i>
RT2R	5'-GTA TCA TCA TAA GTC TTG-3'	<i>gtfB</i>
RT3F	5'-ACA ACA AAC CAG TCT ACA-3'	<i>gtfC</i>
RT3R	5'-CGG ACT GGT TGC TGT ATT-3'	<i>gtfC</i>
gtfB-F241	5'-CAT ACA GTA ACG ACA AGC AGT AGC TCT A-3'	<i>gtfB</i>
gtfB-R539	5'-GTA CGA ACT TTG CCG TTA TTG TCA TA-3'	<i>gtfB</i>
Sm16S-F1250	5'-CCG GTG ACG GCA AGC TAA-3'	16S rRNA
Sm16S-R1319	5'-TCA TGG AGG CGA GTT GCA-3'	16S rRNA
TaqMan probe		
GtfB TaqMan minor-groove-binder	5'-FAM-ATG TTA TTG ATG ATA GCA ATG C-NFQ-MGB-3'	<i>gtfB</i>
16S rRNA TaqMan	5'-FAM-CTC TGA AAG CCG ATC TCA GTT CGG ATT G-TAMRA-3'	16S rRNA

* Endonuclease recognition sequences are shown in bold. FAM, 6-carboxyfluorescein; NFQ, non-fluorescent quencher; MGB, minor-groove-binder; TAMRA, 6-carboxytetramethylrhodamine.

planktonic and sessile cells were added to 5 ml of fresh CDM supplemented with 0.5% sucrose to an OD₅₇₀ value of 0.3. Sodium phosphate buffer (1.0 M) was then added to the suspensions to a final concentration of 50 mM (pH 7.0) about 10 min before the FACS analysis was done.

Confocal laser scanning microscopy (CLSM). Microscopic observations and image acquisition were performed with a CLSM system using LSM 5 PASCAL with LSM 5 PASCAL

software (Carl Zeiss); $\lambda_{\text{excitation}}$ 488 nm and $\lambda_{\text{emission}}$ 505–530 nm were analysed. Biofilms for CLSM analysis were prepared the same way as for the FACS analysis. Briefly, overnight cultures of *S. mutans* 854S and GS-5 grown in CDM supplemented with 0.5% sucrose in 6-well polystyrene plates (Corning) were washed several times with CDM and then fresh CDM was added to the wells. Water-immersion lenses (Plan-NEOF-LUAR 25/0.81 mm; Carl Zeiss) were employed for this study.

Fig. 1. Construction of the streptococcal *gfp* plasmid pAYBG854S. The promoterless *gfp* gene was subcloned into pUC19, resulting in pAYG210. A DNA fragment containing *PgtfB* was obtained by PCR amplification of *S. mutans* GS-5 chromosomal DNA and subcloned into the *Bam*HI–*Xba*I site of pAYBG210 to generate pAYBG310. The *gtfB::gfp* fragment flanked by *Sph*I sites was amplified by PCR from pAYBG310 and subcloned into the *Sph*I site of pDL276 to generate pAYBG754S. An erythromycin cassette was then subcloned into the *Sma*I site of pAYBG754S to generate pAYBG854S. MCS, multiple-cloning site; rep and ori, origin of replication.

RT-PCR analysis. Total RNA was isolated from *S. mutans* by using TRIZOL Reagent (Gibco-BRL), according to the manufacturer's instructions, and treated with RNase-free DNase (DNase I; Gibco-BRL). Reverse transcriptase reactions were performed by using SUPERSRIPT II RNase H⁻ Reverse Transcriptase (Gibco-BRL), according to the manufacturer's instructions. For the RT-PCR experiment, primers were designed from the sequences of the *gtfB* and *gtfC* genes; the primer pairs used are listed in Table 2. PCR amplifications were carried out with *Taq* polymerase (Promega) and the following cycling protocol: one cycle at 94 °C for 5 min followed by 40 cycles at 94 °C for 30 s, 47 °C for 30 s and 72 °C for 1 min. To check for DNA contamination, reverse transcriptase was not present in the negative control reaction.

Oligonucleotide primers and probes for real-time RT-PCR. Oligonucleotide primers and probes for *gtfB* and 16S rRNA (a housekeeping gene used as an internal control) were designed by using Primer Express 1.5 software (PE Biosystems). Fluorogenic probes were used to continuously monitor PCR product formation during PCR. The oligonucleotide probe for 16S rRNA was labelled with a reporter dye (6-carboxy-fluorescein) covalently attached at its 5' end and a quencher dye (6-carboxytetramethylrhodamine) covalently attached at its 3' end. A TaqMan minor-groove-binder probe consisting of a 6-carboxyfluorescein-labelled oligonucleotide at the 5' end and a non-fluorescent quencher–minor-groove-binder complex (*T_m* enhancer) at the 3' end was used as a probe for *gtfB* (Kutyavin *et al.*, 2000). Primers and probes were

synthesized and purified by Applied Biosystems. The primers and probes used for real-time PCR are listed in Table 2; the amplicons generated with these primers were 299 and 70 bp in length for *gtfB* and 16S rRNA, respectively.

Real-time RT-PCR. Total RNA was isolated from planktonic and biofilm cells using TRIZOL Reagent (Gibco-BRL) according to the manufacturer's instructions. Single-stranded cDNA was then synthesized in a reaction mixture containing 1.25 U MultiScribe Reverse Transcriptase μl^{-1} , 0.4 U RNase Inhibitor μl^{-1} , 500 μM of each dNTP, 200 nM antisense primer, 1 $\times$ Reverse Transcription buffer, 5.5 mM MgCl_2 (TaqMan Reverse Transcription Reagents; Applied Biosystems) and 1 μg total RNA at 48 °C for 30 min. To check for DNA contamination, purified total RNA without reverse transcriptase served as a negative control. The resulting cDNA and negative control were amplified by using the TaqMan Universal PCR Master Mix (Applied Biosystems), which contained dNTPs with dUTP, AmpliTaq Gold DNA polymerase, Amperase UNG, optimized buffer and a passive reference dye. For each PCR, a mixture containing 5 ng template cDNA, 1 $\times$ Master Mix, 200 nM of each sense and antisense primer and 250 nM TaqMan probe was placed in a 96-well MicroAmp Optical Reaction Plate with Optical Caps (Applied Biosystems). Amplification and detection of the specific products were performed on an ABI PRISM 7700 Sequence Detection System (PE Biosystems) with the following cycle protocol: one cycle at 50 °C for 2 min and one cycle at 95 °C for 10 min followed by 60 cycles at 95 °C for 15 s and 60 °C for 1 min. The critical threshold cycle (C_t) was defined as the cycle at which the fluorescence became detectable above the background fluorescence, and was inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set with C_t values obtained from amplification of known quantities of cDNA. To check the linearity of the detection system, a cDNA dilution series (1/10, 1/100, 1/1000 and 1/10000) was amplified with primer pairs and probes so that a correlation coefficient could be calculated from the standard curve displaying C_t values. The standard curves were used to transform C_t values to the relative number of cDNA molecules. The quantity of cDNA for *gtfB* was normalized to that of cDNA synthesized from 16S rRNA. The relative amounts of RNA per cell were approximately the same for planktonic and biofilm cells (data not shown).

Statistical analysis. The variables for fluorescent intensities of GFP between biofilm and planktonic cells were assessed by using Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Construction of *PgtfB::gfp* fusions

Initial attempts to construct a p15Aori plasmid harbouring the *gtfB* promoter were unsuccessful. However, the *gtfB* promoter was successfully cloned into pUC19. Therefore, the promoterless *gfp* gene was cloned into pUC19 to yield pAYG210. The isolation of a plasmid containing the *gtfB* promoter has been successful only in high-copy-number plasmids (pUCori or ColE1). A 193 bp segment containing the *gtfB* promoter was inserted 5' to the promoterless *gfp* gene in pAYG210 to generate plasmid pAYBG310. To generate a *Streptococcus-E. coli* shuttle vector that contained the *PgtfB::gfp* fusion, the *PgtfB::gfp* fragment (1.0 kb) was

Fig. 2. Fluorescent microscopy image of *S. mutans* 854S expressing GFP. The strain containing the *gtfB::gfp* shuttle vector is shown exhibiting varying degrees of bright-green fluorescence.

excised from pAYBG310 and cloned into the shuttle vector pDL276, which contains a *Streptococcus* replicon and an *E. coli* high-copy-number origin of replication (*ori*). The resulting plasmid, designated pAYBG754S, was used to transform *S. mutans* GS-5 using selection with 200 μg kanamycin ml^{-1} , but transformants were not isolated. Therefore, the erythromycin cassette from pResEmMCS10 was amplified by PCR and subcloned into pAYBG754S to generate pAYBG854S (Fig. 1). Transformation of *E. coli* with this shuttle vector yielded fluorescent transformants, as detected on agar plates. Transformation of *S. mutans* GS-5 with the plasmid extracted from *E. coli* yielded transformants at a rate of approximately 1 to 1.5×10^4 transformants (μg DNA) $^{-1}$ compared with a rate of 1.5×10^4 transformants (μg DNA) $^{-1}$ for the same plasmid extracted from *S. mutans* 854S. To generate a chromosomal promoter fusion, the *PgtfB::gfp* fragment (1.0 kb) was excised from pAYBG310 and cloned into the suicide vector pZ63 (Sato *et al.*, 1991), which does not replicate in streptococci, to generate pAYBG554 (data not shown). Insertion of this plasmid into the *S. mutans* chromosome following single-cross-over integration during transformation resulted in the generation of cells with a single chromosomal copy of the *PgtfB::gfp* fusion.

Detection of GFP-expressing *S. mutans* cells by fluorescence microscopy

The visualization of GFP expressed by *S. mutans* GS-5 cells harbouring chromosomal or plasmid copies of *gfp* was performed by fluorescence microscopy. The strains were grown anaerobically and analysed directly in CDM supplemented with 0.5% sucrose. In CDM containing 50 mM NaCl, the cells harbouring both a single chromosomal copy and multiple copies of *gfp* were not visibly fluorescent (data not shown). However, in CDM containing 50 mM sodium phosphate buffer (pH 7.0), fluorescence was readily detected in the plasmid-

Fig. 3. CLSM images of biofilm development on a polystyrene surface by *S. mutans* 854S carrying a plasmid which constitutively expresses GFP. (a) Topographic features of biofilm development. Sagittal section (side panels) showing large and small microcolonies linked by intercellular bridges. Dimensions of the regions displayed are $336 \times 336 \mu\text{m}$ (x-y perspectives; square panel) and $336 \times 82 \mu\text{m}$ (x-z perspectives; rectangular panels). (b) Three-dimensional reconstructed renderings of the biofilm. Dimensions correspond to Fig. 3(a).

containing strain (Fig. 2) but not in the strain harbouring the chromosomal copy of *gfp* (data not shown). These results indicated the crucial role of environmental pH on the detection of GFP fluorescence, as noted by Hansen *et al.* (2001).

CLSM of GFP-expressing *S. mutans* cells and biofilm morphology

The biofilm formed by the GFP-expressing *S. mutans* 854S cells that bound to the polystyrene surface of the wells after 24 h cultivation in CDM supplemented with 0.5% sucrose is shown in Fig. 3. After 24 h incubation, the *S. mutans* 854S cells formed several very large amorphous microcolonies and many small microcolonies were interspersed across the biofilm (Fig. 3a). The micrographs of single sections indicated an increase of

Fig. 4. Comparison of flow cytometry analysis of *gfp* expression by *S. mutans* *gtfB* promoters in planktonic and biofilm cells. Flow cytometry histograms of *S. mutans* 854S were analysed. Bars indicate regions of positive fluorescence; the percentage of positive cells is indicated in both panels. Results are representative of four experiments.

fluorescence in the biofilm as a function of sucrose, as did the side panels (zx and zy scans) on the entire stack of images. Vertical gradients of fluorescence through the biofilm were also observed (Fig. 3b).

Flow cytometry analysis of GFP-expressing *S. mutans* cells

GFP expression by *S. mutans* 854S was also examined in harvested planktonic (*S. mutans* 854S-P) cells and in sessile (*S. mutans* 854S-B) cells attached to the polystyrene plates, by using flow cytometry. Planktonic and sessile cultures of untransformed wild-type *S. mutans* GS-5 cells served as negative controls. Cells of *S. mutans* strains 854S-P, 854S-B and GS-5 were subjected to flow cytometry after 24 h anaerobic growth in CDM supplemented with 0.5% sucrose. After 24 h cultivation, $8.55 \pm 0.85\%$ (mean $\pm$ SEM, $n=4$) of the *S. mutans* 854S-P cells were found in the regions of positive fluorescence (Fig. 4). In contrast, $40.80 \pm 0.69\%$ (mean $\pm$ SEM, $n=4$) of the *S. mutans* 854S-B cells were found in the same regions ($P < 0.01$; Fig. 4). Therefore, these results indi-

Fig. 5. Genetic organization of the *gtfBC* gene region and RT-PCR analysis of mRNA transcripts. (a) The orientations of the *gtfB* and *gtfC* genes are indicated by open arrows. The solid arrows indicate the primers used for RT-PCR. (b) Gel electrophoresis of amplified DNA fragments. Lanes: RT1, amplification with primer pair RT1F/RT1R; RT2, amplification with primer pair RT2F/RT2R; RT3, amplification with primer pair RT3F/RT3R. +, Standard amplifications with RT-PCR; –, negative control reactions without reverse transcription.

cated an almost five-fold increase in *gtfB* expression in the biofilm cells relative to the planktonic cells. To check the effects of different sugars on *gtfB* regulation, the GFP expression levels of *S. mutans* 854S cells grown in CDM supplemented with 0.5% sucrose, glucose or fructose were analysed by fluorescence-activated cell sorting. After 24 h anaerobic growth, there were no significant differences in *gtfB* expression between planktonic cells grown in the presence of the three sugars (data not shown). These results suggested that increased *gtfB* expression in the sessile cells relative to the planktonic cells of strain GS-5 was regulated by the environment of the biofilm.

Evidence for transcription of the *gtfBC* locus in *S. mutans*

We performed RT-PCR analysis to identify the transcripts of the *gtfB* and *gtfC* genes of *S. mutans*. A pair of oligonucleotide primers spanning the borders of *gtfB*–*gtfC* (RT1F/RT1R; Table 2) was designed. In addition to RT1F/RT1R, two pairs of primers were designed to amplify *gtfB* (RT2F/RT2R) and *gtfC* (RT3F/RT3R) as controls (Table 2). Fig. 5(b) shows the 507 bp PCR product amplified with the RT1F/RT1R primer pair. No PCR products were observed from total RNA preparations that had not been reverse transcribed first (Fig. 5b), indicating that the RT-PCR products were not derived from contaminating chromosomal DNA.

Quantification of *gtfB* transcription in planktonic and biofilm cells

Real-time RT-PCR (TaqMan) assays were employed to examine the relative abundance of *gtfB*-bearing transcripts in planktonic and biofilm cells. The test samples

Fig. 6. Real-time quantitative RT-PCR analysis of *S. mutans gtfB* expression in biofilm and planktonic cells. (a) Amplification plot of *S. mutans* cDNA. Serial dilutions of cDNA from *S. mutans* 16S rRNA were used as templates for real-time PCR. The horizontal bar indicates the threshold fluorescence ($T=0.116$). A standard curve was generated from the amplification plot ($r^2=0.995$). ○, $0.1 \times$ cDNA; ●, $0.01 \times$ cDNA; □, $0.001 \times$ cDNA; ■, $0.0001 \times$ cDNA. (b) Amplification plot and relative quantities of cDNA from *S. mutans gtfB* in biofilm and planktonic cells, as assessed by TaqMan assays. The amount of *gtfB* cDNA was determined and normalized to the amount of cDNA of 16S rRNA following reverse transcription of total RNA isolated from biofilm and planktonic cells. The mean Ct value of the *gtfB*-specific cDNA from planktonic cells was 41.049 ± 0.521 (mean $\pm$ SEM, $n=4$) and that of the cDNA from biofilm cells was 38.933 ± 0.375 (mean $\pm$ SEM, $n=4$). The horizontal bar indicates the threshold fluorescence, as in (a). △, Planktonic cells; ▲, biofilm cells. The results shown are representative of those obtained from two independently isolated RNA preparations.

were cDNA-primed with an antisense primer, and 10-fold serial dilutions of cDNA from 16S rRNA were employed to generate the standard curves. Kinetic curves were defined by a Ct value that marked the fractional cycle number during the exponential phase at which the fluorescence of a given sample became significantly different from the baseline signal. Kinetic curves are

shown for four concentrations of cDNA (Fig. 6a). The linearity of *gtfB* transcription was exactly the same for the planktonic and the biofilm cells (data not shown). For each cDNA template concentration, a single PCR product of the expected size for primer pair gtfB-F241/gtfB-R539 was detected by gel electrophoresis (data not shown). The *gtfB* mRNA in the planktonic- and biofilm-cell samples was quantified by measuring the Ct and by using calibration curves obtained during the same experiments to determine the quantity of target message. The results shown here are representative of four independent experiments. The same linearity of transcription was observed when the template was *gtfB*-specific cDNA from either planktonic or biofilm cells (data not shown). The mean Ct value of the cDNA from planktonic cells was 41.049 ± 0.521 (mean $\pm$ SEM, $n=4$) and that of the cDNA from biofilm cells was 38.933 ± 0.375 (mean $\pm$ SEM, $n=4$). These experiments revealed that the level of *gtfB* mRNA expressed in biofilm cells was approximately four-fold higher than that expressed in planktonic cells (Fig. 6b).

DISCUSSION

Despite intensive investigations into GTF-encoding genes, the regulation of the expression of these genes has not been thoroughly defined. This is due primarily to the relatively large size of the *gtf* gene products and the high nucleotide sequence similarity of the GTF-encoding genes; both of these factors have made it difficult to perform transcriptional analysis on these genes. If the role of *gtfB* in *S. mutans* biofilm formation is to be defined, detailed transcriptional analysis of *gtfB* is necessary; hence, we initially investigated the feasibility of utilizing *gtfB::gfp* reporter fusions in such studies. Consequently, the work detailed here represents the first report of GFP expression in *S. mutans* using an endogenous promoter to drive *gfp* transcription.

PgtfB::gfp was isolated on high-copy-number plasmids (pUCori or ColE1), but not on a medium-copy-number plasmid (p15Aori) (Y. Nakano, personal communication). Interestingly, the copy number of the plasmid harbouring the *PgtfB* fragment was significantly decreased when compared with that of the original plasmid (data not shown). Therefore, pDL276, a high-copy-number *Streptococcus-E. coli* shuttle vector, was selected for subcloning the *PgtfB::gfp* fusion. Following transformation of pAYBG854S (pDL276 harbouring *PgtfB::gfp*) into *E. coli* DH5 α , GFP expression driven by the *S. mutans* *PgtfB* was active in *E. coli* on agar plates, as revealed by the green fluorescent colonies of the transformants. However, the fluorescence from the *S. mutans* transformants that expressed GFP was not detected on agar plates. In addition to this, the transformation efficiency of *S. mutans* GS-5 with pAYBG854S extracted from *E. coli* was quite low. However, the transformation rate of *S. mutans* GS-5 with pAYBG854S isolated from *S. mutans* 854S was relatively high. This suggested that restriction-modification systems in *E. coli* may be responsible for the

differences seen in transformation efficiency between pAYBG845S isolated from *E. coli* and the same plasmid isolated from *S. mutans* 854S.

For visualization of *S. mutans* 854S cells, CLSM analysis was performed. *S. mutans* harbouring a single chromosomal copy of *gfp* did not display visible fluorescence, but transformants harbouring multiple copies of *gfp* were fluorescent. A study by Acebo *et al.* (2000) reported that fluorescence could be detected from *Streptococcus pneumoniae* cells that harboured a single chromosomal copy of *gfp* by fluorescence microscopy. However, the *gfp* copy number may be one of several important factors affecting the expression of a target promoter. For example, the differences in fluorescence intensity may be influenced by variations in oxygen intake by the bacteria or by other factors (Hansen *et al.*, 2001).

CLSM was also carried out on *S. mutans* biofilms attached to the wells of polystyrene plates. The advantage of using CLSM in biofilm studies is due to the enhanced ability this technique affords for observing and analysing fresh, undisturbed materials in real time (Lawrence & Neu, 1999). From the CLSM images of the *S. mutans* biofilms, it was observed that *gtfB* expression was enhanced on the surfaces of the biofilms; vertical gradients of fluorescence were also observed in the biofilms. In previously utilized flowcell systems, rapid oxygen distribution in *Streptococcus gordonii* biofilms has been confirmed (Hansen *et al.*, 2001). Our results suggest that the oxygen supply may be a limiting factor in the deeper layers of the artificial biofilm. Moreover, the fluorescence pattern of the *S. mutans* biofilm (as seen in Fig. 3a) could be due either to microcolonies where *gtfB* expression is enhanced or to gaps between microcolonies where aeration is better. In the process of bacterial biofilm formation, bacterial species migrate over the colonized surface to form microcolonies and, subsequently, produce an exopolysaccharide matrix to form and support the biofilm (Watnick & Kolter, 2000). Thus, our findings, which suggest enhanced *gtfB* expression in microcolonies, are consistent with this general model for biofilm formation.

Based on the initial experiments detailed here, we have examined *gtfB* expression in planktonic and biofilm cells using two distinct approaches. In earlier work, several groups examined *gtfBC* expression using various reporter systems with a single chromosomal copy of the reporter gene (Burne *et al.*, 1997; Hudson & Curtiss, 1990). It is important to consider several aspects of the promoter-reporter system when analysing the expression of the *S. mutans* *gtfB* and *gtfC* genes. In the present study, we employed GFP expression and flow cytometry to detect the expression of *gtfB* in *S. mutans* biofilms. At first, to eliminate the effects of differences in *gfp* copy number, we compared the ratios of plasmid to chromosomal DNA in biofilm and planktonic cells and found these to be quite similar (data not shown). The fluorescent intensity of the biofilm cells was enhanced compared with that of the planktonic cells. A study by

Hudson & Curtiss (1990) reported that the chloramphenicol acetyltransferase activity of *S. mutans* cells directed by the *gtfBC* operon promoter was approximately two-fold higher in cells bound to saliva-coated beads than in unbound cells. To date, almost all of the transcription analyses done involving the *gtfB* and *gtfC* genes of *S. mutans* and reporter fusions have utilized single chromosomal copies of the reporter genes (Burne *et al.*, 1997; Hudson & Curtiss, 1990). However, a study by Goodman & Gao (2000) indicated that transcription of the endogenous *gtfB* and *gtfC* genes was similar for both chromosomal and plasmid copies of these genes fused to reporters.

A general hypothesis concerning *gtfBC* transcriptional regulation is that a promoter upstream of *gtfB* initiates transcription of a polycistronic message which includes the *gtfB* and the *gtfC* genes (Ueda *et al.*, 1988). However, several groups have reported that there is another promoter in the *gtfB*–*gtfC* intergenic region that might allow the independent expression of *gtfC* (Goodman & Gao, 2000; Smorawinska & Kuramitsu, 1995). Due to difficulties in using Northern-blot analysis, it is reasonable to employ RT-PCR to characterize the transcripts of the *gtfB* and *gtfC* genes. The RT-PCR analysis of the *gtfBC* genes performed here revealed that *gtfC* can be transcribed together with *gtfB* in a single polycistronic mRNA. This finding represents the first experimental verification of this hypothesis. Hence, based upon this result and previous results (Smorawinska & Kuramitsu, 1995), it is suggested that *gtfC* is most likely transcribed from the *gtfB* and the *gtfC* promoters. Therefore, it is likely that earlier studies done using *gtfBC* reporter systems detected not only *gtfB* expression but also *gtfC* expression, since the reporter gene was fused downstream of *gtfC* (Burne *et al.*, 1997; Goodman & Gao, 2000; Hudson & Curtiss, 1990). Therefore, the present results represent the first study of *gtfB* promoter regulation in *S. mutans* biofilms.

To eliminate the effects of *gtfC* expression from our results, we also performed real-time RT-PCR (TaqMan) analysis for only *gtfB* expression in planktonic and biofilm cells. The TaqMan RT-PCR assay is very sensitive and specific for obtaining quantitative information regarding transcription, and the use of this assay represents a significantly different approach for studying *gtfB* expression relative to previous studies. Our analysis demonstrated that the rate of transcription of *gtfB* in biofilm cells was approximately four times higher than that in planktonic cells. These results suggest *gtfB* to be an important factor in *S. mutans* biofilm formation.

The stable expression of GFP by *S. mutans* transformants provides a useful method for studying the interaction(s) of this pathogen in biofilms. The use of CLSM in combination with the *S. mutans* GFP-expressing strain will make it possible to visualize bacterial behaviour in homogeneous and mixed-species biofilms. This approach should accelerate the characterization of

the *S. mutans* *gtfB* gene as a virulence factor and contribute to our understanding of how *S. mutans* behaves in biofilms such as dental plaque.

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