molecular oral microbiology

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A novel phosphotransferase system of *Streptococcus mutans* is responsible for transport of carbohydrates with α-1,3 linkage

D. Ajdic[1,2] and Z. Chen[2]

1 University of Miami, Miller School of Medicine, Miami, FL, USA
2 University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Correspondence: Dragana Ajdic, Department of Dermatology and Cutaneous Surgery, University of Miami, Miller School of Medicine, 1600 NW 10th Avenue, RMSB 2089, Miami, FL 33136, USA Tel.: +1 305 243 0475; fax: +1 305 243 6191; E-mail: dajdic@med.miami.edu

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SUMMARY

The most common type of carbohydrate-transport system in *Streptococcus mutans* is the phosphoenolpyruvate–sugar phosphotransferase system (PTS). Fourteen PTS exist in *S. mutans* UA159. Several studies have shown that microorganisms growing in biofilms express different genes compared with their planktonic counterparts. In this study, we showed that one PTS of *S. mutans* was expressed in sucrose-grown biofilms. Furthermore, the same PTS was also responsible for the transport and metabolism of disaccharide nigerose (3-*O*-α-D-glucopyranosyl-D-glucose). Additionally, the results indicate that the studied PTS might be involved in the transport and metabolism of carbohydrates synthesized by glucosyltransferase B and glucosyltransferase C of *S. mutans*. To our knowledge, this is the first report that shows PTS transport of a disaccharide (and possibly extracellular oligosaccharides) with α-1,3 linkage.

INTRODUCTION

Streptococcus mutans is a major causative agent of human dental caries. The host's diet is very important for the cariogenesis of *S. mutans*, as carbohydrate metabolism by this bacterium plays a key role in the formation of caries. *Streptococcus mutans* is able to metabolize a wide range of carbohydrates that may originate in the diet. If the diet is rich in sugars, especially sucrose, the end product of the sugar metabolism is mostly lactic acid, which can lead to demineralization of tooth enamel. Therefore, sugar transport and metabolism by this bacterium are directly related to the onset and development of dental caries.

The most common type of carbohydrate-transport system in bacteria is the phosphoenolpyruvate–sugar phosphotransferase system (PTS). Each PTS consists of two non-specific energy-coupling components, enzyme I (EI), a heat-stable protein (HPr), and a sugar-specific multiprotein or multidomain permease known as enzyme II (EII). In most cases, subunits IIA and IIB are located in the cytoplasm, while subunits IIC and IID (when present) act as a membrane channel (Postma *et al.*, 1993; Vadeboncoeur & Pelletier, 1997; Deutscher *et al.*, 2006). The source of energy for carbohydrate transport is provided by phosphoenolpyruvate. During the process of carbohydrate PTS transport, the phosphoryl group on phosphoenolpyruvate is transferred to the imported sugar via several PTS proteins. First, the phosphoryl group is transferred to EI. Then, EI transfers the phosphoryl group to a histidine residue on HPr. From HPr the phosphoryl is transferred to EIIA, and then to EIIB. Finally, EIIB phosphorylates carbohydrate as it

crosses the plasma membrane through the transmembrane EIIC, forming sugar-phosphate. The PTS are responsible for binding, transmembrane transport, and phosphorylation of numerous sugar substrates. This holds true for *S. mutans* as well (Abranches *et al*., 2003, 2006; Zeng & Burne, 2009, 2010). We previously showed that 14 PTS exist in *S. mutans* UA159 (Ajdic *et al*., 2002; Ajdic & Pham, 2007).

Following initial colonization of the hard surfaces in the oral cavity, many bacteria including *S. mutans*, synthesize exopolysaccharides (EPS) and exooligosaccrarides (EOS) that result in the formation of a stable biofilm. Both EPS and EOS are produced by the action of extracellular glucansucrases (EC 2.4.1.5), also called glucosyltransferases (GTF) and fructosyltransferase Ftf (EC 2.4.1.10). The GTF are enzymes that catalyse the transfer of glucosyl units formed following the cleavage of sucrose to a growing α-glucose chain, (Henrissat, 1998). The fructose moiety of sucrose is liberated during this process. Similarly, Ftf uses the fructose moiety of sucrose to synthesize the EPS fructan (Gibbons & Houte, 1975; Hamada & Slade, 1980). *Streptococcus mutans* expresses three GTFs: GtfB, GtfC and GtfD (formerly GtfI, GtfSI and GtfS) that produce different sizes and structures of EPS, called glucan. The nature of the linkages between glucosyl units determines their water solubility (Aoki *et al*., 1986; Hanada & Kuramitsu, 1988, 1989). A low content of α-1,3 linkages and high content of α-1,6 linkages is associated with greater solubility of EPS, which is designated as dextran (Kuramitsu, 1975; Walker, 1978; Monchois *et al*., 1999). A reversed proportion of α-1,3 and α-1,6 linkages lead to the formation of water-insoluble glucose polymers called mutan. In addition to glucan production, GTF can transfer glucose to fructose, in which case sucrose isomers are formed (Monchois *et al*., 2000). The EPS produced by extracellular sucrose-using enzymes of *S. mutans* contribute to the pathogenicity (Gibbons & Houte, 1975; Hamada & Slade, 1980; Loesche, 1986; Schroeder *et al*., 1989; Munro *et al*., 1991) of this organism in multiple ways including bacterial adherence (Hamada *et al*., 1984; Schilling & Bowen, 1992) and accumulation on the tooth surface (Freedman & Tanzer, 1982; Larrimore *et al*., 1983), and extracellular storage of carbohydrates that can be used during periods of nutrient deprivation (Wood, 1967; Gibbons, 1968b; Manly & Richardson, 1968; Bowen & Koo, 2011).

Dental plaque, the biofilm that develops on the tooth surface, is a complex community of microorganisms. When the diet of the human host is rich in carbohydrates, the most prevalent organisms present are acidogenic and aciduric bacteria such as *S. mutans* (Bowden *et al*., 1979; Bradshaw *et al*., 1989; Burne, 1998; Bowden, 1999). Several studies have shown that microorganisms growing in biofilms express different genes compared with their planktonic counterparts (Costerton *et al*., 1987, 1995; Burne *et al*., 1997). In this study, we showed that previously uncharacterized PTS (locus SMU.100-105) of *S. mutans* was expressed in sucrose-grown biofilm and in planktonic cultures grown in disaccharide nigerose (3-*O*-α-D-glucopyranosyl-D-glucose). Additionally, the results indicate that this PTS might be also involved in the transport and metabolism of carbohydrates synthesized by glucosyltransferase B (GtfB) and glucosyltransferase C (GtfC) of *S. mutans*. To our knowledge, this is the first report that shows PTS transport of a disaccharide (and possibly EOS) with α-1,3 linkage.

METHODS

Bacterial strains and culture conditions

Streptococcus mutans UA159 was routinely grown in Todd–Hewitt broth with 0.3% yeast extract supplemented, when needed, with antibiotics at the following concentrations: kanamycin (500 μg ml^{-1}), erythromycin (5 μg ml^{-1}). For microarrays and quantitative real-time polymerase chain reaction (qRT-PCR), we used 'chemically defined' medium (CDM) that has been successfully implemented in our previously published studies (Ajdic & Pham, 2007). For RNA isolation, cells were grown in CDM supplemented with ultrapure 0.5% carbohydrate. Lists of the bacterial strains and of the carbohydrates used in this study are presented in Tables 1 and 2, respectively. The environmental conditions used for these experiments were 5% CO_2, 37°C, pH 7.0. As a result of the cell aggregation that occurs in the sucrose-growth medium, the optical density of cultures grown in sucrose was estimated by growing a duplicate culture in CDM supplemented with glucose. All cultures used for qRT-PCR or microarray analyses were grown to a mid-exponential growth phase [optical density at 600 nm (OD_{600}) = 0.65], except when stated otherwise.

Table 1 Bacterial strains used in this study and their derivation

Streptococcus mutans strains	Description	References
WT	UA159, serotype c strain, wild-type	P. Caufield
gtfB$^-$	Δ *gtfB*; Erm^R	This work
gtfC$^-$	Δ *gtfC*; Kan^R	This work
gtfBC$^-$	Δ*gtfBC*; Kan^R	This work
gtfD$^-$	Δ *gtfD*; Kan^R	This work
ftf$^-$	Δ*ftf*; Kan^R	This work
nigB$^-$	Δ*nigB*; Erm^R	This work
gtfB-Cc	*gtfB*; ErmR; *gtfC* constitutive transcription	This work

Erm^R, erythromycin resistance; Kan^R, kanamycin resistance.

Growth curves

Growth curves of *S. mutans* UA159 were generated using a BIOSCREEN C ANALYZER, version 2.4 (Oy Growth Curves AB Ltd., Helsinki, Finland). The cultures were incubated at 37°C for 24–48 h in CDM supplemented with the appropriate carbohydrate. The wideband 420–580-nm filter that is less sensitive to color changes of the media was used to detect the optical density of the cultures.

Biofilm batch cultures

Biofilm batch cultures were grown in polystyrene plates for 48 h in diluted CDM (0.5× CDM) supplemented with 10 mM sucrose. The biofilms were seeded with a 1 : 100 dilution of the 16-h planktonic bacterial culture grown in Todd–Hewitt broth with 0.3% yeast extract and washed twice with phosphate-buffered saline pH 7.4. The environmental conditions used for these experiments were 5% CO_2, 37°C, pH 7.0. The growth medium was replaced with fresh medium after 24 and 3 h before cell collection to ensure expression of carbohydrate transporters (the 3-h incubation was determined empirically). The cells were then collected in TriReagent (Invitrogen, Carlsbad, CA) for RNA isolation.

Construction of the mutants

The mutants *ftf*, *gtfD*, *gtfBC*, *gtfB*, *gtfC*, *gtfB-C^c* and *nigB* were constructed using the Overlap extension PCR method (Chalker *et al.*, 2001). To construct each mutant, approximately 1000-bp upstream and downstream regions [including approximately 100 bp of the 5′-end and 3′-end of each open reading frame (ORF), respectively] was PCR-amplified. A list of the primers used in this study is presented in Table 3. A fragment containing an antibiotic gene (erythromycin – *erm* or kanamycin – *kan* cassette) was amplified with specific primers (Table 3). The three fragments were mixed and amplified with corresponding primers (Table 3). The resulting PCR product was used for transformation of *S. mutans* UA159. Proper construction of the mutants was verified by PCR and sequencing. Nonpolar cassettes were used for construction of the mutants (Zeng & Burne, 2008, 2009), and the transcription of the downstream genes was checked by qRT-PCR.

The *gtfB-C^c* mutant was constructed using the same primers used for the *gtfB*$^-$ mutant (gtfB-P1, P2_erm, P3_erm, P4), except the erm-Fwd-P was used for amplification of the *erm* cassette resulting in the deletion of the *gtfB* and constitutive transcription of the *gtfC* gene. To verify that the *gtfB-C^c* mutant strain was properly constructed, transcription of the *gtfB* and *gtfC* genes was analysed by qRT-PCR and compared with those of the wild-type (WT) and *gtfBC*$^-$ mutants. In the *gtfB-C^c*, the *gtfB* showed no transcription whereas *gtfC* was fully transcribed in aggregation growth mode at OD_{600} 0.65.

Table 2 Disaccharides used in this study

Disaccharide	Units	Bond	Growth	PTS^{Bio} transcription	References
Kojibiose	Two glucose monomers	α(1–2)	No	N/A	This study
Nigerose	Two glucose monomers	α(1–3)	Yes	yes	This study
Maltose	Two glucose monomers	α(1–4)	Yes	No	Ajdic and Pham (2007)
Isomaltose	Two glucose monomers	α(1–6)	No	N/A	This study
Sucrose	Glucose + fructose monomers	α(1–2)	Yes	Yes	This study
Turanose	Glucose + fructose monomers	α(1–3)	No	N/A	This study
Maltulose	Glucose + fructose monomers	α(1–4)	No	N/A	This study
Leucrose	Glucose + fructose monomers	α(1–5)	No	N/A	This study
Isomaltulose	Glucose + fructose monomers	α(1–6)	No	N/A	This study

Table 3 Primers used in this study

gtfB-P1	TAC ATT GAC TCC CCT AAT CTT CTT G
gtfB-P2_erm	TTT TTG TTC ATG TAA TCA CTC CTT CCG CAG TTT ATA ACG CAC TTT CTT GTC
gtfB-P3_erm	ATA ATT CTA TGA GTC GCT GCC GAC TTC AGT TAT TAC GAT GCT AAC TCT GGA
gtfB-P4	GAC CTG ATT ATA TTG AGC AAA GCT G
gtfC-P1	CGT CAT GTT TGA AGG TTT CTC TAA T
gtfC-P2_kan	CGG TAT AAT CTT ACC TAT CAC CTT AGA CTT CCT GAA AGA GAG GTC AAA
gtfC-P3_kan	TCT AAA AGT TCG CTA GAT AGG GTT GGA TTC TTT GAC AAT TTC TTT AGA TT
gtfC-P4	AAA TTC TTG ATT GCT TTT ATT TCC TC
gtfBC-P1	TTT GTG GGA TAG TTT TGT TTT TAT CA
gtfBC-P2_kan	CGG TAT AAT CTT ACC TAT CAC CTT CAC CCA TCT TTT CTT TAC TTT ACG
gtfBC-P3_kan	TCT AAA AGT TCG CTA GAT AGG GTT GGA TTC TTT GAC AAT TTC TTT AGA TT
gtfBC-P4	AAA TTC TTG ATT GCT TTT ATT TCC TC
gtfD-P1	AAA TAT GCT GTT CTT TTT GCT AAC G
gtfD-P2_kan	CGG TAT AAT CTT ACC TAT CAC CTC CAG TGC TTT TTA ACC TTG TAC ATT
gtfD-P3_kan	TCT AAA AGT TCG CTA GAT AGG GTA ACA TGG TTT ACA ACA AAG TCG TC
gtfD-P4	TCC ACT GAA TAA TTT CAC CTA CCT C
ftf-P1	GAA AAA GCT CAT CCA GAT ATT TTC A
ftf-P2_kan	CGG TAT AAT CTT ACC TAT CAC CTC CCA AAA TTT CCC TTT CTT ATA CAT
ftf-P3_kan	TCT AAA AGT TCG CTA GAT AGG GAT TAG CTC TTT TCA GTG CTT TCT GT
ftf-P4	TAG GAA TTA GCC GAC CTT CTT ATT T
bioB-P1	TTA TGA AGA TGC TCT TGA ATG TAT CG
bioB-P2_erm	CAA ATC AAA CAA ATT TTG GGC CCG GTC TTT GAT CTA CTC TAG CCT CAA CAA
bioB-P3_erm	ATA ATT CTA TGA GTC GCT GCC GAC TTC AAT ATA TTC CGG ATG ATT CTG TAA
bioB-P4	TAT ATA ATG CTG AAC CCA TGA ACA TC
erm-Fwd-P	CCG GGC CCA AAA TTT GTT TGA TTT
erm-Fwd	GAA GGA GTG ATT ACA TGA ACA AAA A
erm-Rev	AGTCGGCAGCGACTCATAGAAT
kan-Fwd	AGG TGA TAG GTA AGA TTA TAC CG
kan-Rev	CCC TAT CTA GCG AAC TTT TAG A

erm-Fwd-P: polymerase chain reaction amplification with this primer included erm promoter; erm-Fwd (no P): polymerase chain reaction amplification with this primer did not included erm promoter.

RNA extraction and cDNA synthesis for microarray analysis

RNA extraction and complementary DNA (cDNA) synthesis were performed as previously published by our group (Ajdic & Pham, 2007). Briefly, the cultures for RNA isolation were grown at 37°C to an OD_{600} of approximately 0.65 in CDM containing 0.5% of the desired sugar. The cells were disrupted with a bead beater and the RNA was purified using the Ambion RiboPure™ (Ambion Life Technologies, Grand Island, NY)-Bacteria Kit. Isolated RNA was treated with DNAse I, and the absence of DNA was confirmed by PCR. Complementary DNA was generated from 10 μg RNA with SuperScript II reverse transcriptase (Invitrogen). DNA was fragmented with DNAse I using our previously published method (Ajdic & Pham, 2007). Quality and size (50–200 nucleotides) of the fragmented cDNA was evaluated using a Bioanalyzer 6000 (Agilent Technologies, Santa Clara, CA) followed by labeling with Biotin-ddUTP using the Bio-Array Terminal Labeling Kit (Enzo Life Sciences, Farmingdale, NY).

Quantitative real-time PCR

Quantification of specific transcript was accomplished by the comparative C_T method using the Bio-Rad (Hercules, CA) MyiQ Real-time PCR Detection System. The primers amplified 100–110-bp-specific fragments. Efficiency of amplification was confirmed by analysing melting curves of each amplicon. All qRT-PCR reactions were performed using SYBR Green master mix (Bio-Rad) with specific primers and cDNA (which was prepared following the protocol for cDNA preparation for microarray except, in this case, DNAse I fragmentation and biotin labeling were omitted). Samples in which SuperScript II was omitted during cDNA synthesis were used as a negative control. The qRT-PCR amplification with primers to the *gyrA* was used for normalization. Non-template controls were included to confirm the absence of primer–dimer formation. All samples, including non-template controls, were performed in triplicate.

The comparison of gene expression in various conditions was accomplished by the comparison of C_T values automatically generated by the MyiQ software (Bio-Rad). The relative comparison of expression of the gene of interest (*goi*) in two different conditions (*goi1* and *goi2*) was presented as fold change. All fold change values of the genes of interest were normalized to *gyrA* because our previous data showed that it produced little variation of expression in mid-exponential growth phase (OD_{600} = 0.65) (Ajdic & Pham, 2007). Previously published mathematical models (Pfaffl, 2001; Malke *et al*., 2006) for relative quantification of qRT-PCR data were performed.

Expression microarray

A whole genome custom GeneChip Antisense Expression Microarray chip, designed in collaboration with Affymetrix (Santa Clara, CA), was used in this study. The technical details about the chip were previously published (Ajdic & Pham, 2007).

Hybridization, washing and scanning of the microarray chips were performed according to the procedures described by Affymetrix (www.affymetrix.com) and our previously published protocols (Ajdic & Pham, 2007). The GeneChip® Operating Software (GCOS) version 1.4 analysis program (Affymetrix) was used to analyse gene expression and expression clustering, respectively. The data were compared using GCOS batch analyses. Normalization of all probe sets was carried out by GCOS. The software computed a normalization value: Trimmed Mean $Signal_{baseline}$ = (Normalization Value) × (Trimmed Mean $Signal_{experiment}$). The cut-off score for the analysis was two-fold. Relevant microarray data was confirmed by qRT-PCR.

Microarray accession numbers

Microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under platform and series accession numbers GPL4769 and GSE35605, respectively.

RESULTS

Global transcriptional analysis of *S. mutans* sugar transporters in sucrose-grown biofilm

In a previous study, we determined global differential transcription profiles of *S. mutans* UA159 for 13 carbohydrates when in a planktonic mode of growth (Ajdic & Pham, 2007). In this study, differential expression patterns of carbohydrate transporters were analysed in *S. mutans* biofilms grown in sucrose for 48 h. Transcription profiles of the biofilm grown cells were generated by microarrays and compared with our previous microarray results for planktonic cells (Ajdic & Pham, 2007). The transcription patterns of two carbohydrate transporters were significantly different in the biofilm compared with those in planktonic grown cultures. The genes (SMU.1957–1961) encoding $PTS^{Fru/Man}$ specific for fructose and mannose, and a locus consisting of six genes (SMU.100–105), organized as a putative operon, were upregulated in biofilms (Fig. 1 and Fig. 2). Annotation data (Ajdic *et al.*,

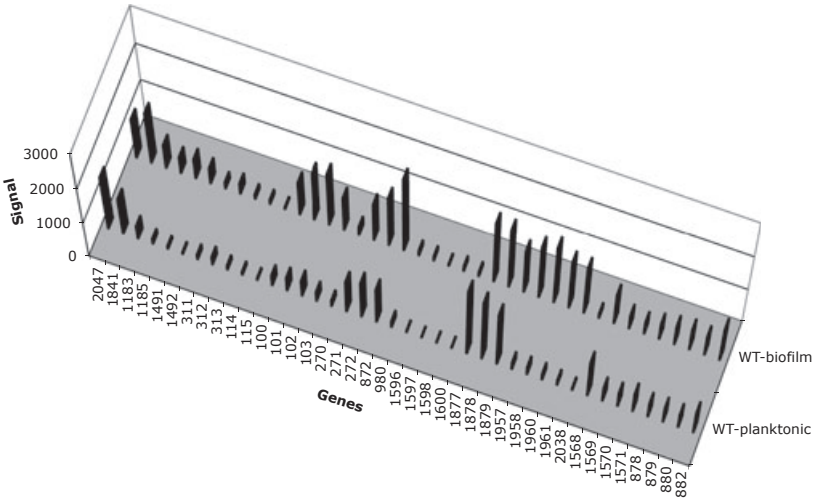

Figure 1 Differential transcription of the genes for phosphotransferase system (PTS) Enzyme II and ABC transporters following growth of *Streptococcus mutans* UA159 in biofilm. Microarray results are presented as vertical bars. The numerical open reading frame designation (as presented in the NCBI genome database) for genes encoding sugar transporters is presented on the *x*-axis. Genes encoding PTS^{Bio} are numbered as 100–103. Complete open reading frame legend is presented in Data S1. Value of the normalized expression signal is presented on the *y*-axis. Transcription of the gene *gyrA* (SMU.1114) served as an endogenous control because it did not show significant variation of transcription in the conditions compared. Two or more biological replicates were analysed in each experiment for each condition, so we present the average results. Conditions: WT-planktonic, wild-type cells grown in 'chemically defined' medium (CDM) culture supplemented with 28 mM glucose; WT-biofilm, cells grown in depleted CDM supplemented with 10 mM sucrose.

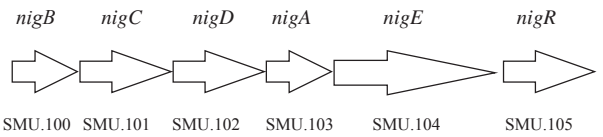

Figure 2 Schematic presentation of putative operon (O^{Bio}) encoding proteins for PTS^{Bio} and metabolic enzymes. Arrows represent the genes. Genes *nigB*, *nigC*, *nigD* and *nigA* encode putative EIIB, EIIC, EIID and EIIA components of the PTS^{Bio}, respectively. Gene *nigE* encoding an enzyme (annotated as putative α-glucosidase/glycosyl hydrolase) and a transcriptional regulator (*nigR*) are also part of this putative operon. The numerical open reading frame designation (as deposited in NCBI genome database) for genes encoding PTS^{Bio} is presented below the arrows.

2002) suggested that this latter putative operon encoded enzymes for PTS transport and metabolism of an unknown substrate. Because the genes of this locus were transcribed in sucrose-grown biofilm, we named the putative operon O^{Bio} and the PTS it encodes PTS^{Bio}.

Transcription of the PTS^{Bio} was also studied in glucose-grown or fructose-grown biofilms using qRT-PCR. *Streptococcus mutans* formed very fragile biofilm if grown in defined medium supplemented with sugars other than sucrose. Expression of the PTS^{Bio} was very low in glucose-grown or fructose-grown biofilms (see Fig. S1) compared with the same in sucrose-grown biofilms. Specifically, its transcription was approximately five-fold lower in glucose-grown and seven-fold lower in fructose-grown biofilm. Therefore, the PTS^{Bio} may not play an important role in biofilm metabolism in the absence of sucrose.

Computational analysis of the O^{Bio}

The O^{Bio} locus was analysed using two ORF-calling programs, Glimmer (Salzberg *et al.*, 1998; Delcher *et al.*, 1999) originally used for annotation of the *S. mutans* UA159 genome database and the National Center for Biotechnology Information ORF finder (www.ncbi.nml.nih.gov). Results revealed the presence of six ORFs organized in a putative operon. Domain and motif search for the predicted ORFs suggested the presence of a four-component transport system of the PTS family encoding EII subunits (EIIB, EIIC, EIID and EIIA), an enzyme involved in carbohydrate metabolism, and a transcriptional regulator of the *Lac*I repressor family. The putative operon structure is shown in Fig. 2. The PTS subunits consisted of EIIB belonging to the PTS_IIB_ mannose superfamily (cd00001; pfam03830), a cytoplasmic component of PTS necessary for the uptake of carbohydrate across the cytoplasmic membrane and its phosphorylation; EIIC of the EII_sorbose superfamily (pfam03609), a membrane subunit; EIID of the AGA superfamily (mannose/fructose/sorbose family) (pfam03613); and EIIA of the mannose superfamily (pfam03610), a cytoplasmic subunit that receives a phosphoryl group from HPr and transfers it to EIIB, which phosphorylates the substrate. The putative enzyme showed high similarity to α-glucosidase/glycosyl hydrolases (cd06595; pfam02065) of the GH31 superfamily. Enzymes of this family cleave a terminal carbohydrate moiety from a substrate that varies in size, depending on the enzyme. The transcriptional regulator contained specific structural features that included a small DNA-binding domain with a helix-turn-helix motif at the N-terminus (cd01392). Analysis of the C-terminus revealed a regulatory ligand-binding domain for oligomerization and for effector binding, and an approximately 18-amino-acid linker connecting these two functional domains (cd06291). For *Lac*I-like transcriptional regulators, the ligands are sugars.

Expression of the PTS^{Bio} is coordinated with EPS/EOS production

Cultures of *S. mutans* grown in defined medium supplemented with sucrose exhibit cell-to-cell aggregation. The aggregation is promoted by the extracellular EPS/EOS produced by *S. mutans*. We will refer to this mode of growth as 'aggregation growth mode'. The transcription of the genes encoding the PTS of UA159 was analysed by microarrays following this growth condition (Fig. 3). The genes encoding the PTS^{Bio} were transcribed in aggregation growth mode (Fig. 3). However, when the cultures were grown in the defined medium supplemented with sucrose and dextranase, sucrose-induced cell aggregation was abolished. The genes encoding the PTS^{Bio} were not transcribed in the presence of dextranase (Fig. 3). So it was evident that the genes encoding the PTS^{Bio} responded to the formation of EPS/EOS produced from sucrose. Hence, sucrose might not be the primary substrate for the PTS^{Bio}.

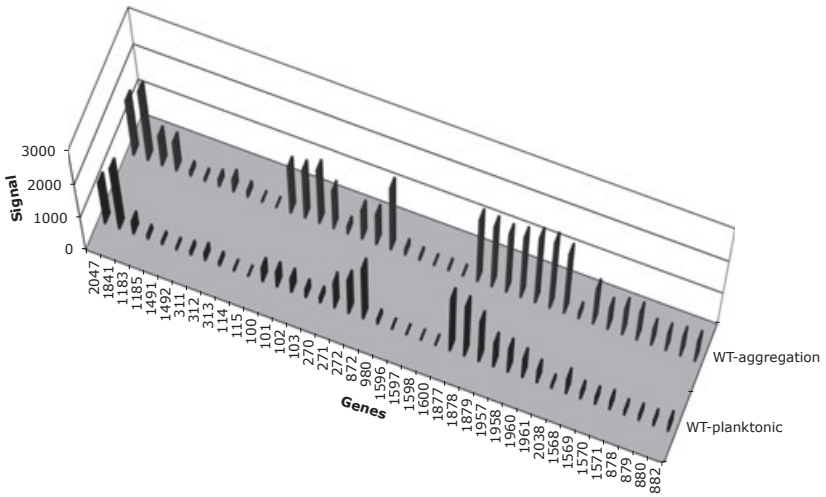

Figure 3 Differential transcription of the genes for phosphotransferase system Enzyme II and ABC transporters following growth of *Streptococcus mutans* UA159 in sucrose. Description of the microarray data presentation is explained in Fig. 1. Conditions: WT-planktonic, wild-type cells grown in 'chemically defined' medium (CDM) supplemented with 0.5% sucrose and 10 U ml^{-1} dextranase; WT-aggregation, cells grown in CDM supplemented with 0.5% sucrose.

GtfB and GtfC are also required for PTS^{Bio} expression

Streptococcus mutans produces three glucosyltransferases (GtfD, GtfB and GtfC encoded by *gtfD*, *gtfB* and *gtfC*, respectively) and one fructosyltransferase (Ftf encoded by *ftf*) that are responsible for synthesis of glucans and fructans, respectively. Glucans can be water-soluble or water-insoluble (Kuramitsu, 1975; Walker, 1978; Monchois *et al*., 1999). Water-soluble glucans and fructans are considered to be extracellular sugar storage (Wood, 1967; Gibbons, 1968b; Manly & Richardson, 1968). Because PTS^{Bio} was expressed in sucrose-dependent biofilm and sucrose-dependent aggregation growth mode we hypothesized that its expression was induced by oligosaccharides and polysaccharides synthesized from sucrose moieties (glucose and fructose). To test our hypothesis, genes encoding enzymes responsible for water-soluble glucan or fructan synthesis were deleted from the genome of UA159. Specifically, single *gtfD*$^-$ and *ftf*$^-$ mutants were constructed by replacing these genes with non-polar antibiotic cassettes (as stated in Methods). Transcription of the first gene (*nigB*) of the O^{Bio} was analysed in the mutants using qRT-PCR and compared with that of the WT, following an aggregation mode of growth. The results revealed minor change of the *nigB* transcription in either mutant (1.4- and 1.3-fold difference in *ftf*$^-$ and *gtfD*$^-$ single mutants, respectively) compared with the WT (Fig. 4A). Data suggested that the activity of GtfD and Ftf was not required for the PTS^{Bio} transcription.

Our next question was whether expression of the Gtfs responsible for production of water-insoluble glucans was necessary for transcription of O^{Bio}. To answer this question, a *gtfBC*$^-$ double mutant was constructed using antibiotic replacement strategy. Again, transcription of the *nigB* was analysed in this mutant using qRT-PCR and compared with that of the WT following an aggregation mode of growth. Transcription of *nigB* was 10.8-fold lower in the *gtfBC*$^-$ mutant (Fig. 4A) indicating that either GtfB or GtfC or both were required for expression of the PTS^{Bio}. Finally, to find out if one or both of these enzymes contributes to the expression of the studied transporter, their genes were individually replaced with antibiotic cassettes resulting in construction of the *gtfB*$^-$ and the *gtfC*$^-$ mutants. Transcription of the *nigB* was analysed in both mutants using qRT-PCR. As shown in Fig. 4A, transcription of the *nigB* was 3.3-fold lower in *gtfB*$^-$ and 6.7-fold lower in *gtfC*$^-$ compared with that of the WT, indicating that the products of these enzymes contribute to the differential regulation of the *nigB*.

To analyse if additional carbohydrate transporters were differentially transcribed in the *gtfB*$^-$, *gtfC*$^-$ and *gtfBC*$^-$ mutants, microarray analysis was performed. As shown in Fig. 4B, transcription patterns for the genes encoding carbohydrate transporters were comparable in the mutants compared with that of the WT,

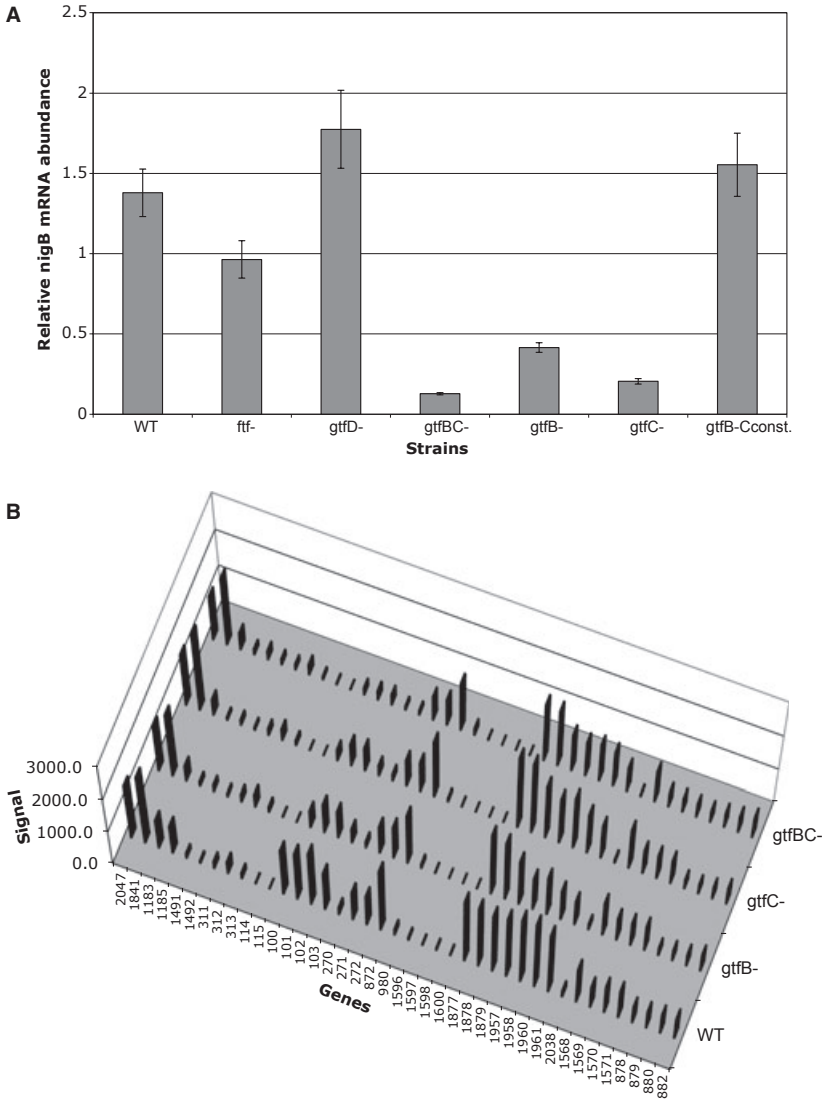

Figure 4 (A) Transcription of the *nigB* in the wild-type (WT) and *ftf* and *gtf* mutants. Gene transcription was analysed by quantitative real-time polymerase chain reaction (qRT-PCR) in the WT and the mutant strains following an aggregation mode of growth. Messenger RNA (mRNA) was isolated from the tested strains during a mid-exponential phase of growth (OD_{600} 0.65). The real-time PCR results were presented as relative mRNA abundance (vertical columns). Standard deviation is presented for each result. (B) Differential transcription of the genes for phosphotransferase system Enzyme II and ABC transporters following growth of *gtfB*$^{-}$, *gtfC*$^{-}$ and *gtfBC*$^{-}$ mutants. Description of the microarray data presentation is explained in Fig. 1.

except for the genes encoding PTS^{Bio}, which were highly transcribed only in the WT.

Constitutive transcription of the *gtfC* triggers transcription of O^{Bio}

Several attempts to clone *gtfB* and *gtfC* genes on a plasmid for complementation of the corresponding deleted genes failed. Plasmid constructs exhibited deletions upon transformation, which resulted in considerably lower or complete lack of gene expression. To circumvent this problem, a genomic transcriptional fusion of the *gtfC* was constructed. In this construct, the *gtfB* gene was deleted from the genome and the *gtfC* gene was expressed from the constitutive *erm* promoter (Claverys *et al*., 1995). This mutant was named *gtfB-C*^{C}. To verify proper transcription of the *gtfB* and *gtfC* genes in the constructed strain, qRT-PCR was performed as explained in the Methods. Transcription of the *nigB* was analysed in

the *gtfB-C*^{C} mutant by qRT-PCR following aggregation growth mode (OD_{600} 0.65). The results showed that *nigB* transcription was similar to that of the WT (Fig. 4A), suggesting that the constitutive transcription of the *gtfC* in the absence of *gtfB* was sufficient to induce full transcription of the O^{Bio} in the mid-exponential phase of growth.

Substrate for the PTS^{Bio} accumulates in aggregation mode of growth and in biofilm

Cell aggregates form quickly at OD_{600} 0.25–0.3 during the cultivation of the WT in CDM supplemented with sucrose. However, aggregation of the *gtfB*$^{-}$ and *gtfC*$^{-}$ mutants was delayed compared with that of the WT. The *gtfC*$^{-}$ mutant formed visible aggregates at OD_{600} 0.35–0.4, whereas aggregation of the *gtfB*$^{-}$ mutant cells was apparent around OD_{600} 0.4–0.45. No aggregation was noticed in the *gtfBC*$^{-}$ mutant cells throughout growth. Because of the variation in timing of the glucan production in the mutants and the WT, we have also analysed transcription of the *nigB* in the late stage (OD_{600} 0.9) of the aggregation-growth mode by qRT-PCR. As presented in Fig. 5, the *nigB* transcription was similar in the *gtfB*$^{-}$ (1.2-fold difference) and slightly lower in the *gtfC*$^{-}$ mutant (1.57-fold difference) compared with that of the WT following this growth condition. However, transcription of the *nigB* was more than four-fold lower in the *gtfBC*$^{-}$ double mutant.

Additionally, transcription of all UA159 carbohydrate transporters (including the PTS^{Bio}) was analysed in the *gtfB*$^{-}$ and *gtfC*$^{-}$ mutants grown in biofilms. This analysis was performed by microarrays and the results for selected genes (SMU.100–103) were confirmed by qRT-PCR (data not shown). Results showed that the transcriptional profiles of the studied genes in the mutants were similar to that of the WT following biofilm growth (see Fig. S2). Furthermore, expression of the PTS^{Bio} was also analysed by microarrays in sucrose-grown biofilms of the WT in which the biofilms were incubated in fresh medium supplemented with glucose during the last 3 h of biofilm growth. Transcription of the PTS^{Bio} in glucose-incubated biofilms was similar to that of the sucrose-incubated biofilms (see Fig. S2).

Collectively, these data suggest that the substrate (s) for the PTS^{Bio} accumulate in the presence of sucrose, GtfB and GtfC. We can also conclude that the accumulation of substrate(s) in the presence of sucrose and in the absence of one glucosyltransferase (GtfB or especialy GtfC) is delayed.

What is the substrate for PTS^{Bio}?

The GTFs of different microorganisms can produce sucrose isomers such as leucrose, turanose, maltulose and isomaltulose (Table 3; Monchois *et al.*, 2000). We hypothesized that GtfB and GtfC of UA159 might be able to produce sucrose isomers that could

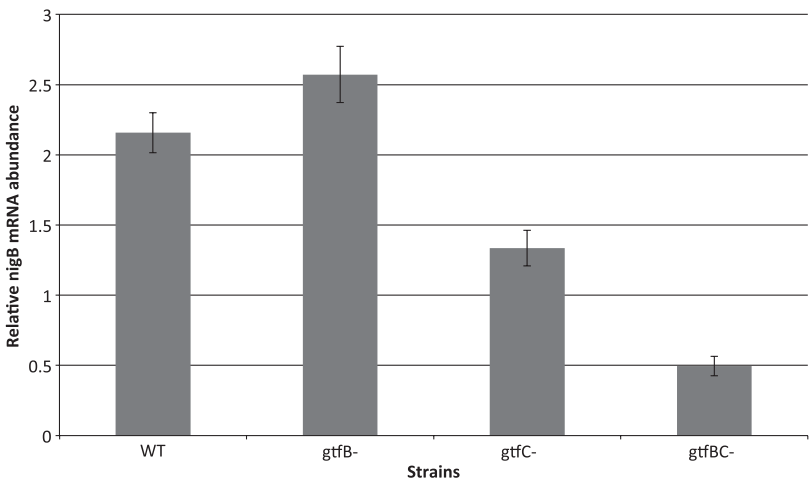

Figure 5 Transcription of the *nigB* in the *gtfB*$^{-}$ and *gtfC*$^{-}$ mutants during late exponential phase of aggregation mode of growth. All samples were collected at an OD_{600} = 0.9. Gene transcription was analysed by quantitative real-time polymerase chain reaction (qRT-PCR). Transcription of the *nigB* in the wild-type (WT) and the *gtfBC* mutant was used as the positive and negative control, respectively. The real-time PCR results were presented as relative messenger RNA (mRNA) abundance (vertical columns). Standard deviation is presented for each result.

be taken-up by the PTS^{Bio}. Therefore, UA159 was grown in leucrose, turanose, maltulose or isomaltulose as a sole carbohydrate source. The results revealed that UA159 did not grow in CDM supplemented with any of these sucrose isomers, suggesting that they were neither substrate for the PTS^{Bio}, nor for any other carbohydrate transporter of this strain.

As mentioned previously, GTFs of *S. mutans* produce α-linked glucose EOS and EPS from sucrose. The majority of the linkages found in the EPS and EOS are α-1,6 and α-1,3 although others, such as α-1,4 and α-1,2 can also be present (Monsan *et al*., 1995; Khalikova *et al*., 2005). Because our data showed that activity of GtfB and GtfC is required for expression of the PTS^{Bio}, we hypothesized that this transporter could be responsible for the uptake of disaccharides or oligosaccharides containing glucose molecules linked by the above-mentioned bonds. To check this, growth of UA159 and transcription of the PTS^{Bio} were analysed in CDM supplemented with 0.5% disaccharides consisting of two glucose molecules linked by the bonds found in EOS produced by *S. mutans*. Disaccharides chosen for this analysis were: kojibiose α-1,2, nigerose α-1,3, and isomaltose α-1,6 (Table 3). Maltose, a disaccharide in which glucose molecules are linked by an α-1,4 bond, was excluded from the analysis because our previous data showed that maltose supported growth of UA159, but the PTS^{Bio} was not expressed in the presence of maltose (Ajdic & Pham, 2007). The strain UA159 neither grew in kojibiose nor in isomaltose, but it did grow in nigerose. Furthermore, *nigB*, the first gene of the putative operon encoding the PTS^{Bio} was transcribed following growth in nigerose, as shown by qRT-PCR (Fig. 6A). To find out if additional carbohydrate transporters of UA159 were differentially transcribed following growth in nigerose, microarray analysis was performed. As shown in Fig. 6B, transcription patterns for the genes encoding carbohydrate transporters were similar following growth in glucose or nigerose, except for the genes encoding PTS^{Bio}, which were highly transcribed in nigerose only. Additionaly, maltodextrin and multiple sugar ABC transport systems were also slightly upregulated, which may indicate that they were also involved in nigerose uptake. Considering that both ABC transporters are primarily responsible for oligosacharide transport, an alternative explanation might be that nigerose serves as a primer for synthesis of oligosaccharides, which are then transported via these two ABC systems.

Deletion of *nigB*

Deletion of any gene encoding an EII component of PTS results in an inactive transport system. Therefore, to verify the phenotype of the PTS^{Bio} we deleted the gene *nigB* encoding the EIIB component of the PTS^{Bio}. The mutant $nigB^{-}$ was constructed using an overlap extension PCR method (as explained in Methods). The $nigB^{-}$ retained capacity to grow in glucose, but lost capability to grow in nigerose (Fig. 7). These data clearly show that the *nigB* gene is required for cell growth in the presence of nigerose.

DISCUSSION

In a previous study, we determined global differential transcription profiles of *S. mutans* UA159 for several monosaccharides, disaccharides, a β-glucoside, oligosaccharides and a sugar-alcohol (Ajdic & Pham, 2007). Our results revealed that PTS were responsible for transport of most carbohydrates tested. In fact, all monosaccharides, disaccharides, β-glucoside and sugar-alcohols we tested were transported by PTS. In contrast, two ABC transporters were specific for oligosaccharides. All of these experiments were performed in planktonic cultures. In this study, differential expression patterns of carbohydrate transporters were analysed in *S. mutans* biofilms and compared with those of planktonic cultures. Two carbohydrate transporters, $PTS^{Fru/Man}$ and PTS^{Bio} (and accompanying enzymes) were differentially expressed in sucrose-grown biofilms compared with those in sucrose-grown or glucose-grown planktonic cultures. Our previous studies showed that the $PTS^{Fru/Man}$ (SMU.1957–1961) was fully induced in planktonic cultures grown in fructose or mannose (Ajdic & Pham, 2007). The same PTS was partially induced in planktonic cultures grown with sucrose in the presence of exogenously added dextranase (Ajdic & Pham, 2007). In this study, we showed that the $PTS^{Fru/Man}$ was upregulated in biofilms and aggregation growth mode. The $PTS^{Fru/Man}$ transports fructose and mannose (Ajdic & Pham, 2007; Zeng & Burne, 2010). Consequently, its induction was expected following growth in sucrose because sucrose serves as a substrate for

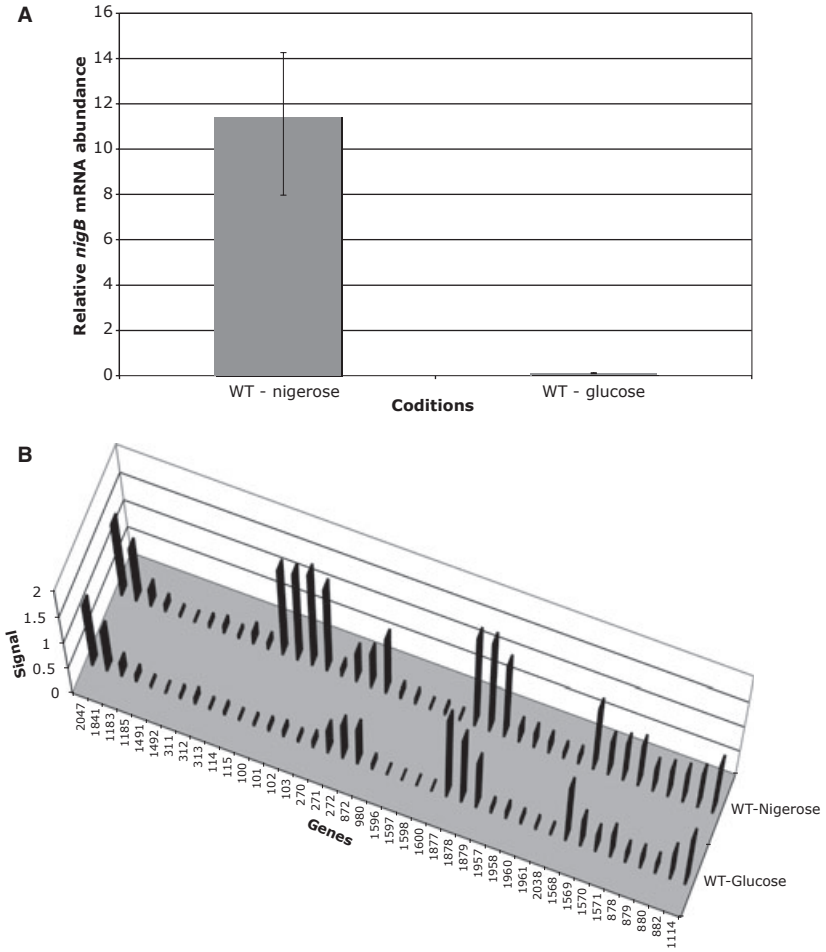

Figure 6 (A) Transcription of the *nigB* following growth in nigerose. Cells were grown in planktonic culture to mid-exponential phase (OD_{600} 0.65) in 'chemically defined' medium supplemented with nigerose or glucose. Gene transcription was analysed by quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR results were presented as relative messenger RNA (mRNA) abundance (vertical columns). Standard deviation is presented for each result. (B) Differential transcription of the genes for phosphotransferase system Enzyme II and ABC transporters following growth of UA159 in nigerose or glucose. The same complementary DNA (cDNA) samples analysed by qRT-PCR were used for microarrays. Description of the microarray data presentation is explained in Fig. 1.

glucosyltransferases (Hamada & Slade, 1980), fructosyltransferase (Hamada & Slade, 1980) and fructanase (Dacosta & Gibbons, 1968; Walker *et al.*, 1983). These extracellular enzymes of *S. mutans* release the fructose moiety from sucrose, which is then taken up by fructose PTS (PTS^{Fru} and $PTS^{Fru/Man}$). Interestingly, expression of the $PTS^{Fru/Man}$ was much higher in biofilm and aggregation growth mode than in planktonic cultures grown in sucrose with dextranase, suggesting a lower concentration of fructose in the media during planktonic growth of *S. mutans*, perhaps because of a lower rate of fructose moiety liberation from sucrose in the presence of a high concentration of dextranase. Some microorganisms synthesize extracellular dextranases and *S. mutans* is one of them (Guggenheim & Burckhardt, 1974). Dextranases catalyse the hydrolysis at the α-1,6-glycosidic bond of dextran and release of glucose or short oligosaccharides consisting of several glucose molecules (Walker, 1972; Khalikova *et al.*, 2005). The activity of the *S. mutans* dextranase is apparently well synchronized with the production of other enzymes involved in extracellular sugar metabolism resulting in the accumulation of dextrans if sucrose is available. However, in the presence of a high concentration of dextranase added to the media at the beginning of the culture growth, newly synthesized dextran is rapidly cleaved (Walker, 1972; Hamada *et al.*, 1975), which

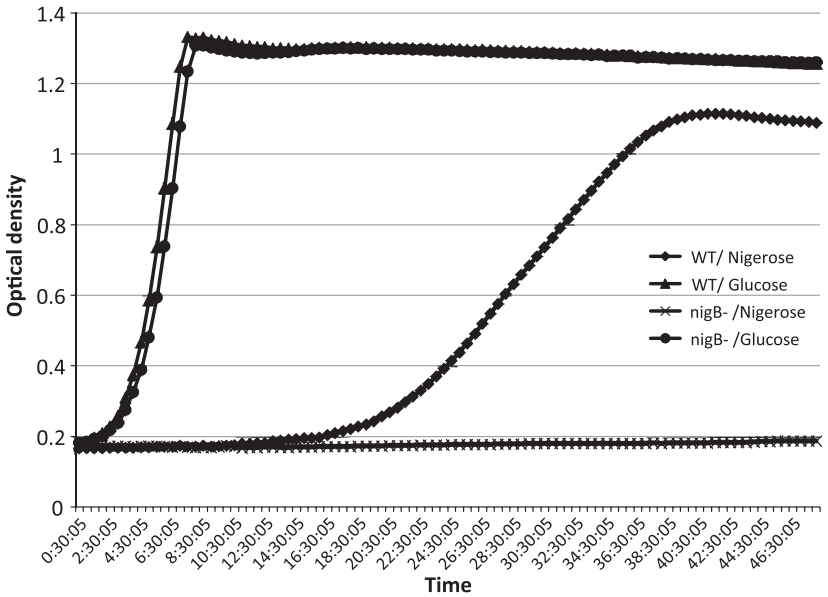

Figure 7 Growth of the wild-type (WT) and the *nigB*$^{-}$ mutant in nigerose or glucose. Samples were grown at 37°C for 48 h in 'chemically defined' medium supplemented with 0.5% sugar.

may result in unbalanced production of glucosyltransferase, fructosyltransferase and fructanase and consequently lower liberation of fructose from sucrose and lower expression of inducible PTS$^{Fru/Man}$.

The second transporter that exhibited full expression in biofilm and aggregation growth mode was PTS^{Bio}. Computational analysis of the O^{Bio} (SMU.100–105) suggested that this putative operon encoded proteins for PTS transport, metabolism and regulation of carbohydrate use. Our previous studies showed that the PTS^{Bio} was not expressed in planktonic cultures grown in 13 different sugars (Ajdic & Pham, 2007). Furthermore, PTS^{Bio} was not expressed in sucrose-grown planktonic cultures in the presence of exogenously added dextranase when this enzyme was added at the initial stage of growth. Oral glucans contain a large proportion of α-1,3-glycosidic bonds that are resistant to the action of dextranase. However, if dextranase is added at the initial stage of the glucan synthesis, this enzyme is able to suppress EPS production because initially the oligosaccharide chains are elongated only by the attachment of glucosyl residues with α-1,6-glycosidic linkages (Walker, 1972). Our results showed that if EPS production was suppressed by dextranase (despite the presence of sucrose), PTS^{Bio} was not expressed. The result led us to conclude that PTS^{Bio} was not responsible for sucrose uptake in this experimental condition. Furthermore, we hypothesized that *S. mutans* used sucrose to synthesize an appropriate substrate that is then taken up by PTS^{Bio}. The *S. mutans* expresses four extracellular enzymes responsible for EPS synthesis: three glucosyltransferases (GtfD, GtfB and GtfC enzymes; EC 2.4.1.5) and fructosyltransferase (Ftf enzyme; EC 2.4.1.10). The GtfD synthesizes EPS in which the prevalent bonds are α-1,6-glycosyl linkages (glucans) (Hanada & Kuramitsu, 1989), whereas the GtfB and GtfC produce EPS rich in α-1,3-glycosyl linkages (mutans) (Aoki *et al.*, 1986; Hanada & Kuramitsu, 1988). The Ftf is responsible for production of EPS (fructans) composed predominantly of α-2,1-linked fructosyl units (Ebisu *et al.*, 1975; Birkhed *et al.*, 1979). Because α-1,6-glycosyl linkages of glucan and α-2,1-fructosyl linkages of fructan can be cleaved to oligosaccharides by dextranase and fructanase respectively, these two types of EPS are considered extracellular carbohydrate storage (Wood, 1967; Gibbons, 1968a,b; Manly & Richardson, 1968) and as such, possible substrate for the PTS^{Bio}. However, neither deletion of the *ftf* gene nor deletion of the *gtfD* gene showed significant effect on expression of PTS^{Bio}. Surprisingly, simultaneous deletion of the *gtfB* and *gtfC* genes completely abolished PTS^{Bio} transcription in the presence of sucrose during mid and late-exponential phases of aggregation growth mode. Unfortunately, we could not

analyse expression of the PTS^{Bio} in the double *gtfBC*$^{-}$ mutant in biofilm because this mutant does not form biofilm. Independent deletion of either *gtfB* or *gtfC* dramatically decreased transcription of this PTS in the exponential phase of culture growth, suggesting that the GtfB and GtfC use sucrose to synthesize the substrate that is then taken up by the PTS^{Bio}. Furthermore, our results showed that if either of these enzymes was expressed, full transcription of the PTS^{Bio} was detected in the later stages of aggregation and biofilm growth, suggesting that either enzyme was capable of synthesizing the substrate, albeit with different timing. It is also possible that the modification of the substrate of one enzyme by the other produces the optimal carbohydrate molecule for which the PTS^{Bio} exhibits the highest affinity. Another interesting observation was expression of the PTS^{Bio} in the 48-h biofilm even if sucrose was replaced with glucose during the last 3 h of biofilm growth, suggesting that the substrate for this transporter accumulated in biofilm. Furthermore, transcription of O^{Bio} was not inhibited by glucose under studied experimental conditions. Therefore, most likely, O^{Bio} is not catabolically repressed by glucose. This is further supported by the absence of the CRE consensus sequence in the promoter region of the O^{Bio}.

What is the substrate for the PTS^{Bio}? We tested a series of disaccharides to address this question. Tested disaccharides (maltose, kojibiose, nigerose and isomaltose) consisted of glucose moieties linked by α-glycosyl bonds found in *S. mutans* EPS. In addition, we also tested several sucrose isomers (leucrose, turanose, maltulose and isomaltulose) known to be produced by Gtf of different organisms. UA159 did not grow in disaccharides with α-1,6 (isomaltose) and α-1,2 (kojibiose) glycosyl linkages, nor did it grow in any of the sucrose isomers tested. In contrast, this strain grew in maltose (α-1,4 linkage) and maltotriose (Ajdic & Pham, 2007; Webb *et al*., 2008), but PTS^{Bio} was not expressed in the presence of these sugars (Ajdic & Pham, 2007). Among all tested sugars, nigerose (α-1,3 linkage) was the only disaccharide capable of inducing O^{Bio}. Furthermore, deletion of the *nigB*, the first gene of O^{Bio}, rendered the mutant incapable of growth in nigerose, confirming that this sugar is a substrate for the PTS^{Bio}. To our knowledge, this is the first report showing that a disaccharide in which two glucose units are linked by α-1,3 bond is used for growth by *S. mutans*. Our results suggest that disaccharide (and possibly oligosaccharides) consisting of α-1,3-linked glucose molecules synthesized by GtfB and GtfC from sucrose are substrates for the PTS^{Bio}, and as such serve as extracellular carbohydrate storage in *S. mutans* biofilm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Annotation of genes for carbohydrate transport and metabolisam in *S. mutans* UA159.

Figure S1. Transcription of the *nigB* following biofilm growth in glucose, fructose or sucrose.

Figure S2. Differential transcription of the genes for phosphotransferase system (PTS) Enzyme II and ABC transporters following growth of *gtfB*$^-$ and *gtfC*$^-$ mutants in biofilm.

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