Global Transcriptional Analysis of *Streptococcus mutans* Sugar Transporters Using Microarrays

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Abstract

The transport of carbohydrates by *Streptococcus mutans* is accomplished by the phosphoenolpyruvate-phosphotransferase system (PTS) and ATP-binding cassette (ABC) transporters. To undertake a global transcriptional analysis of all *S. mutans* sugar transporters simultaneously, we used a whole-genome expression microarray. Global transcription profiles of *S. mutans* UA159 were determined for several monosaccharides (glucose, fructose, galactose and mannose), disaccharides (sucrose, lactose, maltose and trehalose), a β-glucoside (cellobiose), oligosaccharides (raffinose, stachyose and maltotriose) and a sugar alcohol (mannitol). The results revealed that PTSs were responsible for transport of monosaccharides, disaccharides, β-glucosides and sugar alcohols. Six PTSs were transcribed only if a specific sugar was present in the growth medium, thus they were regulated at the transcriptional level. These included transporters for fructose, lactose, cellobiose, trehalose, and two transporters for mannitol. Three PTSs were repressed under all conditions tested. Interestingly, five PTSs were always highly expressed regardless of the sugar source used, presumably suggesting their availability for immediate uptake of most common dietary sugars (glucose, fructose, maltose and sucrose). The ABC transporters were found to be specific for oligosaccharides, raffinose, stachyose and isomaltosaccharides. Compared to the PTS, the ABC transporters showed higher transcription under several tested conditions, suggesting that they might be transporting multiple substrates.
Numerous studies have implicated *Streptococcus mutans* as the principal causative agent of human dental caries. It is well known that host diet is important for *S. mutans* cariogenicity, and sugar metabolism of this bacterium plays a key role in the formation of caries. *S. mutans* is able to metabolize a wide range of carbohydrates that may originate from dietary sources or from host macromolecules. If the diet is rich in sugars, especially sucrose, the end product of sugar metabolism is mostly lactic acid that 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.

In bacteria, sugar substrates are taken up by ABC (ATP-binding cassette) transporters, galactoside-pentose hexuronide (GPH) translocators and most commonly by phosphoenolpyruvate (PEP)-sugar phosphotransferase systems (PTS). The bacterial PTSs are responsible for the binding, transmembrane transport, and phosphorylation of numerous sugar substrates. These systems are also involved in the regulation of a variety of metabolic and transcriptional processes (30, 38). The PTS consists of two non-specific energy-coupling components, Enzyme I (EI) and a Heat-stable protein (HPr), as well as several sugar specific multiprotein permeases known as Enzyme II (EII). In most cases, enzymes IIA and IIB are located in the cytoplasm, while enzyme IIC acts as a membrane channel (38). Fourteen PTS systems have been found in *S. mutans* UA159 based on sequence analysis (4).

The ABC transporters are widespread in bacteria and show a common global organization with three types of molecular components. Typically in Gram-positive bacteria, an ABC transporter consists of two integral membrane proteins (permeases), two peripheral
membrane proteins that bind and hydrolyze ATP, and an extracytoplasmic solute-binding receptor that can be a lipoprotein anchored to the external surface of the cytoplasmic membrane or a cell surface-associated protein that is bound to the external membrane surface via electrostatic interactions. Four sugar ABC transport systems exist in *S. mutans* (4), including the well described multiple sugar metabolism (MSM) system (29, 34, 35).

GPH translocators are electrochemical-potential driven transporters and they catalyze the uptake of sugars in symport with monovalent cations (H\(^+\), Na\(^+\) or Li\(^+\)). In different bacterial species, these transporters exhibit specificity for β-galactosides, lactose and both anomers of galactose (27). Most members of the GPH family are regulated by PTS (25). PTS transport of galactose has not been reported in *S. mutans* and it is possible that this sugar is transported via an ABC or a GPH transporter.

In this study, we analyzed differential transcription profiles of *S. mutans* genes involved in sugar transport, metabolism and regulation following growth in thirteen different sugars: glucose, fructose, mannose, sucrose, lactose, galactose, maltose, maltotriose, trehalose, raffinose, stachyose, cellobiose, and mannitol. Although sugar transport and metabolism by *S. mutans* have been extensively studied, this is the first report in which transcriptional analysis of all sugar transporters of this organism was performed simultaneously using whole-genome microarrays.

**Materials and Methods**

**Bacterial strain and media.** The strain used in this study was *S. mutans* UA159. Bacterial cultures were routinely propagated at 37°C in Todd-Hewitt Broth and 0.3 % Yeast Extract (THY). For RNA isolation, cultures were grown overnight in THY, pelleted, washed two times
in phosphate buffer saline (PBS) and re-suspended in defined medium (FMC) (36) supplemented with 0.5% carbohydrate (~99-99.5% purity, Sigma, except for stachyose and maltotriose that were >98% and >96%, respectively and purchased from Fluka). Dextranase (1,6-α-D-glucan 6-
glucanohydrolase) from Penicillium sp. (Worthington) was added, when indicated, to the glucose- and sucrose-grown cultures of UA159 in a concentration of 10 U/ml.

Growth curves of *S. mutans* UA159 were generated using a Bioscreen C Analyzer, version 2.4 (Oy Growth Curves AB Ltd., Finland) that kinetically and simultaneously measured the development of turbidity of multiple cultures. The cultures were incubated at 37°C for 24 hours in defined medium (FMC) supplemented with the appropriate carbohydrate. The wideband 420-580 filter that is rather insensitive to color changes of the media was used to detect the optical density of the cultures. Growth of *S. mutans* in most of the studied sugars showed similar growth rates with the exception of growth in cellobiose, mannose and mannitol. However, growth of UA159 in each of these three sugars was similar to one another and so the microarray results were compared to each other and to the glucose-grown samples. Growth of UA159 on galactose was considerably slower as compared to any other growth condition.

**RNA isolation.** Each *S. mutans* culture was inoculated from a single colony in 5 ml of THY and incubated at 37°C for approximately 16 hours. The culture was pelleted and washed three times with defined media (FMC). The cells were then resuspended in 5 ml of FMC containing 0.5% of the desired sugar. This culture was transferred to 45 ml of pre-warmed FMC also supplemented with 0.5% sugar and grown at 37°C to an OD_{600} of 0.62 to 0.65. The pellet was collected by centrifugation (6000 rpm) at 4°C for ten minutes. One half of the cell pellet was used for RNA isolation and processed as four samples. The samples were resuspended in 400 μl of ice cold RNAwiz (Ambion) and disrupted with 250 μl of 0.1mm Zirconia Beads using a bead beater.
Cells were subjected to three consecutive 1-minute homogenizations. Two sample lysates were pooled and purified using the Ambion RiboPure™-Bacteria Kit according to the provided protocol. Isolated RNA was treated twice with DNase I (Ambion) to remove traces of chromosomal DNA. After the treatment, RNA samples were cleaned with the Qiagen RNeasy® MinElute™ Cleanup Kit. The purity, concentration, and quality of RNA samples were confirmed by PCR, NanoDrop spectrophotometer measurements, and gel electrophoresis.

**cDNA synthesis.** For cDNA synthesis, 15 mg of RNA was mixed with 1.25 mg of random primers, incubated at 70°C for 10 minutes, and then 25°C for 10 minutes. The reverse transcription was performed in 1x First Strand Buffer supplemented with 10 mM DTT, 0.5 mM dNTP Mix, 60 U of RNase inhibitor (RNaseOut, Invitrogen), and 1500 U of SuperScript II Reverse Transcriptase (Invitrogen) in a final volume of 60 ml. The reaction was incubated at 25°C for 10 minutes, 37°C for 1 hour, and 42°C for 1 hour. Reverse transcription was terminated by a 15 minute incubation at 70°C. To remove residual RNA, the cDNA samples were treated with 0.2 N NaOH at 65°C for 30 minutes and then neutralized with 0.2 N HCl before being purified with the Qiagen MinElute™ PCR Purification Kit. Synthesized cDNA was quantified using a NanoDrop spectrophotometer. cDNA was fragmented with Roche DNAse I (0.06 U per 1 mg of cDNA) at 37°C for 10 minutes. Inactivation of the enzyme was performed at 98°C for 10 minutes. Quality and size (50-200 nucleotides) of the fragmented cDNA was evaluated using Bioanalyzer 6000 (Agilent Technologies) followed by labeling with Biotin-ddUTP using the BioArray Terminal Labeling Kit (Enzo).

**Microarray.** A whole genome custom GeneChip Antisense Expression Microarray was designed in collaboration with Affymetrix (Santa Clara, CA). On the oligonucleotide array, each gene of *S. mutans* was represented by seventeen 25-mer oligonucleotides that were designed to
be complementary to the target sequence. These oligonucleotides served as unique, sequence-specific detectors (termed: perfect match probe). An additional control element on these arrays was the use of seventeen mismatch (MM) control probes for each gene. These probes were designed to be identical to their perfect match (PM) partners except for a single base difference in the central position. The presence of the mismatched oligonucleotide allows cross-hybridization and local background to be estimated and subtracted from the PM signal. A probe pair is called positive when the intensity of the PM probe cell is significantly greater than that of the corresponding MM probe cell, and a probe pair is called negative if the situation is reversed. All probes were designed using Affymetrix probe-selection software and were selected to assure minimal cross-hybridization. Each probe was tiled to the array in approximately one million copies.

Hybridization, washing and scanning of the microarray chips were performed according to the procedures described by Affymetrix (www.affymetrix.com). The hybridization solution contained 100 mM MES, 1 M NaCl, 20 mM EDTA and 0.01% Tween 20, pH 6.6 (referred to as 1 X MES). In addition, the solution contained 0.1 mg ml\(^{-1}\) herring sperm DNA, 0.5 mg ml\(^{-1}\) BSA, 7.8% DMSO and 50 pM of the B2 control oligo. Samples were placed in the array cartridge and hybridization was carried out at 47°C for 16 hours with mixing on a rotisserie at 60 rpm. The hybridization solution was removed and the array washed and stained in the Fluidics station using FlexMidi_euk2v3_450 protocol with modifications for \(P.\ aeruginosa\) array (as recommended in the technical manual Affymetrix Inc, Santa Clara, CA). To enhance the signals a 10 \(\mu\)g ml\(^{-1}\) streptavidin and 2 mg ml\(^{-1}\) BSA in 1 X MES solution was used as the first staining solution. After the streptavidin solution was removed, an antibody mix was added as the second stain containing 0.1 mg ml\(^{-1}\) Goat-IgG, 5 \(\mu\)g ml\(^{-1}\) anti-streptavidin antibody and 2 mg ml\(^{-1}\) BSA.
in 1 X MES. Nucleic acid was fluorescently labeled by incubating with 10 µg ml\(^{-1}\) streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and 2 mg ml\(^{-1}\) BSA in 1 X MES. The arrays were read at 570 nm with a resolution of 0.5 µm using a laser scanner 7G (Affymetrix, Santa Clara, CA).

The GeneChip® Operating Software (GCOS) version 1.4 analysis program (Affymetrix, Santa Clara, CA) was used for the analysis of gene expression and expression clustering, respectively. The data were compared using GCOS batch analyses. Normalization of all probe sets was done by GCOS. The software computes a normalization value so that: Trimmed Mean Signal\(_{\text{baseline}}\) = (Normalization Value) \times (Trimmed Mean Signal\(_{\text{experiment}}\)). The cut off score for the analysis was two fold. Due to a limitation of the software, differences lower than two-fold could be inconsistent and therefore not significant.

Microarray data is 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 GSE6973, respectively.

**Real-Time (RT) PCR.** Quantification of the specific transcript was accomplished by the comparative C\(_T\) method using the Bio-Rad (Hercules, CA) MyiQ Real-time PCR Detection System with fluorescein-spiked SYBR Green as the fluorophore. Binding of the dye to the DNA leads to an increase in fluorescence and is the basis for the detection of the specific cDNA PCR product. The cycle at which the fluorescence intensity generated by a specific PCR product passes a fixed threshold is defined as the threshold cycle (C\(_T\)). The primers for RT-PCR analysis were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The length of the primers was 23-24 nucleotides, the melting temperature was 60°C and they amplified 100-110 bp specific fragments. Efficiency of
amplification was confirmed by analyzing melting curves of each amplicon following the amplification with template dilution over four orders of magnitude. The efficiency of amplification was between 97.6 and 111.7% for each primer pair, showing that the amplicons were generated with similar efficiency. Primers used in this study are listed in Table S12.

All RT-PCR reactions were performed in 1X SYBR Green master mix (Bio-Rad) with specific primers (2 ng/µl) and 0.4 ng/µl of cDNA sample in a 25ml volume. The RT-PCR protocol included one cycle at 95°C for 90 seconds, followed by 40 cycles with two steps each at 95°C for 15 seconds and at 60°C for 1 minute. The RT-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 run in triplicates. The cDNA samples used as templates for RT-PCR reactions were identical to those used in microarray experiments.

The comparison of gene expression in various conditions was accomplished by comparison of the C_T values that were automatically generated by the MyiQ software. The relative comparison of expression of the gene of interest (goi) in two different conditions (goi1 and goi2) was presented as fold change (FC). All FC values of the genes of interest were normalized to gyrA since it produced little variation of expression. Previously published mathematical models (19, 23) for relative quantifications of RT-PCR data were performed. Briefly, the ΔC_T was calculated by subtracting the gyrA C_T values from the goi C_T values. Calculation of ΔΔC_T involved subtraction of the ΔC_T goi2 value from ΔC_T goi1 value. The FC value was calculated using formula FC = 2^{-ΔΔCT}. Correlation analysis of RT-PCR and microarray data sets was performed using Excel.
Results

Previous computational analyses of the *S. mutans* UA159 genome database showed the presence of fourteen PTS systems and four ABC transporters for probable transport of sugars and sugar alcohols (4). Additional analysis confirmed the presence of three carbohydrate ABC transporters with one of them (SMU.827-828) possibly being a carbohydrate exporter involved in cell-wall biogenesis (42). The fourth ABC transporter (SMU.1118-1120) was proven to be responsible for ribonucleoside uptake (40). Consequently, the latter two ABC transporters were not analyzed in this study.

Global transcriptional patterns were obtained following growth on different carbohydrates including monosaccharides (glucose, mannose, fructose and galactose), disaccharides (sucrose, lactose, maltose and trehalose), a β-glucoside (cellobiose), oligosaccharides (raffinose, stachyose and maltotriose) and a sugar-alcohol (mannitol). To assure reproducibility of the data, the cultures were always grown to a similar optical density. We have also observed that the growth phase of the cultures had a profound effect on the data obtained from pair-wise comparison. Because of this, a growth curve of UA159 was generated for each condition tested (Fig. S1, Supplemental Material) and the samples that exhibited similar growth were compared (see Materials and Methods). Additionally, all samples were also compared to glucose-grown cultures.

A total of 48 samples (ten biological replicates each for glucose and fructose, and two to four replicates for every other tested sugar) were prepared. The microarray results are presented in Tables S1-S11 (Supplemental Material). The results generated for each biological replicate of each condition were compared to the results for glucose-grown samples or another relevant
condition, and several tables were generated for each trial and for each of the studied sugars. All
tables representing results for each condition were compared and consistent changes in gene
expression were compiled. For example, pair-wise comparisons of two glucose- and two
fructose-grown samples showed differential expression in 266, 337, 376 and 401 genes when all
possible combinations were performed. Among these genes, 22 showed more than a 2-fold
decrease and 46 genes showed a 2-fold or greater increase in transcription. However, all four
paired treatment conditions revealed 13 common genes with decreased and 10 genes with
increased transcription (highlighted, Table S1) in glucose compared to fructose. Similar
comparisons were performed for all conditions (Tables S2-S11).

In the presence of fructose, one operon (SMU.1961, mannose/fructose-specific EIIA;
SMU.1960, mannose/fructose-specific EIIB; SMU.1958, mannose/fructose-specific EIIC;
SMU.1957, mannose/fructose specific EIID; SMU.1956, part of the mannose/fructose-specific
PTS) (Fig. 2) encoding a putative EII, showed dramatically increased transcription with an
induction level of 5- to 11-fold for different genes of the operon (Table S1, Fig 3B, Fig. 4A)
suggesting that this is an inducible fructose EII. Microarray results showed a 11-fold increased
transcription for ORF SMU.1956 in the presence of fructose, suggesting that this gene, initially
annotated as a hypothetical protein, is an integral part of the operon and probably part of EII for
the inducible fructose PTS.

The operon characterized in this study as an inducible fructose operon (SMU. 1956-1961)
(Fig. 2) was also differentially transcribed in the presence of mannose. The genes of this operon
were induced 14 to 37-fold in the presence of mannose as compared to mannitol grown cells
(Table S2, Fig. 4A). Therefore, this EII is an inducible fructose and mannose EII that we named
EII^{Fru/Man}. This is the first study that demonstrates the existence of an inducible mannose transporter.

When strain UA159 was grown in the presence of sucrose and dextranase, genes for three sugar-transporters exhibited differential expression; i.e., the genes for maltose/maltodextrin ABC transporter (SMU.1568-1571; Fig. 2) showed a 2 to 3-fold decrease, the genes for raffinose ABC transporter (SMU.878-882; Fig. 2) also exhibited a 2 to 3-fold decrease and the genes for an inducible EII^{Fru/Man} (Fig. 2) showed an approximate 2-fold increase in transcription as compared to the glucose and dextranase-grown cells (Table S3, Fig. 4A).

In the presence of lactose, the transcription of two transporter genes, SMU.1491 (EIIIBC) and SMU.1492 (EIIA) (Fig. 2) was increased 15- and 24-fold, respectively (Table S4, Fig. 4B). These two genes are an integral part of the lactose-tagatose operon (14-16, 28) and they are also annotated as a putative EII^{Lac}, therefore we conclude that this is a major lactose transporter. All genes for the lactose-tagatose 6-phosphate and Leloir pathway were also induced (Table S4). Additionally, ORFs SMU.1488 and SMU.1489 were highly induced in lactose-grown cells (Table S4). These two genes are located next to the gene for phospho-β-galactosidase, the last gene of the lactose operon. Their elevated transcription in lactose suggests that they are also involved in lactose and/or galactose metabolism. The same genes that were induced in lactose also exhibited differential transcription in galactose grown cells (Table S5, Fig. 4B).

Three of the *S. mutans* transporters were differentially expressed in raffinose-grown cells. Genes encoding an ABC transporter (SMU.878-880 and 882, Fig. 2) which are a part of the MSM operon showed a 2.5 to 3.5-fold increase in transcription, whereas transcription of the genes for the EII^{Fru/Man} (Fig. 2) were increased 4-11 fold (Table S6, Fig. 4B). Interestingly, transcription of the genes for EII^{Lac} (Fig. 2) was elevated more than 6- and 9-fold, respectively.
In addition to differential transcription of the genes for transport, raffinose-grown cells exhibited differential transcription in several metabolic operons: lactose-tagatose, galactose and the MSM operon (Table S6).

The same transporters and catabolic operons induced following growth in raffinose were also induced in stachyose grown cells (Table S7, Fig. 4B). This result was not surprising because stachyose is an oligosaccharide very similar to raffinose and the only difference between the two is an additional galactose moiety in stachyose.

In the presence of trehalose, two genes of the putative trehalose operon were differentially transcribed as compared to other conditions. The gene for putative EII\textsuperscript{Treu} (SMU.2038) was found to have more than a 22-fold increase in transcription (Table S8, Fig. 4C). Also, transcription of the gene for a putative trehalose-6-phosphate hydrolase (SMU.2037) was increased more than 18-fold.

Transcription of the operon for an ABC transporter (SMU.1568-1571, sugar-binding protein, two permease components, ATP-binding protein, respectively) (Fig. 2) was 5-fold increased in the presence of maltose (Table S9) and 4-fold increased in the presence of maltotriose (Table S10), suggesting its involvement in maltose and maltotriose transport (Fig. 4C).

The genes for EII\textsuperscript{Cel} locus (SMU.1596-1601) (Fig. 2) were differentially transcribed with an induction level being 11 to 42-fold higher for different genes of the same operon over its basal level of transcription (Table S11, Fig. 4C). The gene SMU.1597, originally annotated as a “conserved hypothetical protein” was induced 52-fold. Further analysis of its deduced amino acid sequence demonstrated membrane protein characteristics (data not shown), suggesting its involvement in cellobiose transport.
In the presence of mannitol, 12 genes distributed in two loci were differentially transcribed. The first locus included seven genes (SMU.308, sorbitol/mannitol-6-phosphate dehydrogenase; SMU.309, regulator of sorbitol/mannitol operon; SMU.310, sorbitol/mannitol operon activator; SMU.311, PTS sorbitol/mannitol-specific EIIC2; SMU.312; PTS sorbitol/mannitol-specific EIIBC; SMU.313, PTS sorbitol/mannitol-specific EIIA; SMU.314, part of sorbitol/mannitol regulon) and was reannotated as the sorbitol/mannitol operon (Fig. 2). The induction level of different genes of the sorbitol/mannitol operon varied between 10 and 16-fold (Tables S2 and S11, Fig. 4C). The second locus was a well-characterized mannitol operon (SMU.1185, PTS mannitol-specific EIIBC; SMU.1184, transcripational regulator; SMU.1183, PTS mannitol-specific EIIA; SMU.1182, mannitol-1-phosphate dehydrogenase) (Fig. 2). Transcription of the different genes of this operon was between 4 and 10-fold higher in mannitol as compared to cellobiose (Tables S2 and S11, Fig. 4C).

Transcription of selected sugar-transporter genes in different conditions was also tested using Real-Time (RT) PCR. Specific genes for sugar-transport (for ABC transporters, gene for solute-binding receptor; for PTS, one of the genes for EII) were selected for this analysis. The microarray and RT-PCR results were compared and although the RT-PCR results revealed higher fold-change range as compared to microarray data, these two data sets showed a correlation coefficient of 0.92 for data presented in log2 scale (Fig. 5). The RT-PCR data revealed a 3.8-fold (log2=1.9) increased transcription of scrA, a 3.2-fold (log2=1.7) increased transcription of SMU.872 (encoding EII\textsuperscript{Fru}) and a 2.8-fold (log2=1.5) decreased transcription of SMU.2047 (encoding EII\textsuperscript{Mal/Glu}) in the presence of sucrose (Fig. 5). RT-PCR data also revealed a 3.7-fold (log2=1.9) and 2.6-fold (log2=1.4) elevated transcription of one PTS (SMU.2047) in the presence of maltose and maltotriose, respectively (Fig. 5).
Discussion

Global differential transcription profiles were obtained for growth of *S. mutans* in thirteen different sugars using microarrays. The results achieved for all samples grown in the same sugar showed excellent reproducibility, as presented in Fig. 2 for glucose- and fructose-grown samples. Nevertheless, selected microarray results were verified using RT-PCR and these two data sets showed an excellent correlation (Fig. 5).

Microarray results indicated that fructose was transported by PTS in *S. mutans*. In fact previous studies indicated that fructose was transported into the cell via an inducible and a constitutive PTS (11, 41). Wen and colleagues (41) have characterized transcription of both transporters. Comparison of both published gene clusters and their flanking regions to the genome database (4) revealed that the EII for these PTSs were designated as SMU.872 (EII\textsubscript{Fru}, constitutive) and SMU.113-116 (inducible). The microarray results confirmed that SMU.872 was constitutively expressed (Fig. 3B and 4A). However, our previous (24) and current transcriptional analysis revealed a different fructose induced operon (SMU.1956-1961) (Fig. 3B and 4A). Recently published data (43) concerning fructose metabolism confirmed our results. The previously reported fructose inducible operon (SMU.113-116) (41) showed very low transcription in fructose-grown cells. Furthermore, this operon exhibited low expression in every condition tested in this study. It is not completely clear why this operon was not transcribed in the presence of fructose. One explanation could be that there is a mutation in the promoter region that hinders operon transcription in the tested strain. Alternatively, this may not be a fructose EII, but an EII for some unknown sugar.
Growth in sucrose is inherently difficult due to dextran (glucan) production. Strain UA159 formed visible aggregates when grown in the presence of sucrose and consequently it was difficult to obtain an accurate optical density of the culture. Since the growth rate of the culture and the number of the cells in each sample compared was critical for microarrays, we could not perform microarray experiments following growth in sucrose. To address this question we have analyzed sucrose transport of UA159 grown in a culture supplemented with extracellular endodextranase. This enzyme catalyzes the endohydrolysis at the $\alpha$-1,6-glycosidic bond of dextran (glucan) and releases short isomaltosaccharides consisting of 3-5 glucose molecules. *S. mutans* is also known to produce an extracellular endodextranase that cleaves dextran (12). The activity of this enzyme 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, when the cells were grown in the presence of a high concentration of dextranase, dextran was cleaved and aggregation of *S. mutans* was prevented.

Induction of the EII$^{\text{Fru/Man}}$ following growth in sucrose was expected because sucrose serves as a substrate for fructosyltransferase (13) and fructanase (8, 39). These extracellular enzymes release the fructose moiety from sucrose, which is then taken up by its specific PTS. Although fructose is the most probable substrate for this PTS, we cannot rule out the possibility that this PTS also transports sucrose.

It is not completely clear why there was decreased transcription of the maltodextrin ABC transporter (SMU.1568-1571) in the presence of sucrose and dextranase, since one would expect that this condition would favor production of isomaltosaccharides. The high concentration of dextranase added to the cultures possibly lowered the concentration of longer isomaltosaccharides (maltodextrins). Therefore, longer isomaltosaccharides might be a substrate
for this ABC transporter and their lower concentration in the culture might result in its decreased transcription. Alternatively, since the control samples (glucose and dextranase grown cultures) did not contain products of hydrolyzed glucans, it is possible that this transporter exhibits some specificity for glucose.

It was surprising to find a decreased transcription of the raffinose ABC transporter (SMU.878-882) upon growth in sucrose given that it was previously reported that this transporter takes up multiple sugars, including sucrose, in strain LT11 (17, 29, 35). Our microarray data demonstrated that the transcription of the raffinose transporter is very low in sucrose as compared to any other sugar tested (Fig. 4). This result suggested that neither the raffinose nor maltodextrin ABC transporter was involved in sucrose transport under the conditions tested. Thus, sucrose is not an inducer of the two ABC operons. However, it is possible that upon their induction by the specific substrates, these transporters may play a minor role in sucrose transport.

Several transporters in *S. mutans* take up sucrose, and among them is the best characterized, high affinity sucrose PTS. The gene for EII\textsuperscript{Suc} is located in an operon that also includes the gene for sucrose-6-phosphate hydrolase (31). Therefore, sucrose is converted to sucrose-6-phosphate during transport into the cell and is subsequently hydrolysed to glucose-6-phosphate and fructose. A Blast search of the nucleotide sequence for the EII\textsuperscript{Suc} gene (*scrA*) revealed that this gene was designated as SMU.1841 in the genome database (4). The *scrA* gene was consistently highly transcribed under every condition tested in this study. However, the RT-PCR data revealed increased transcription of *scrA* in the presence of sucrose (Fig. 5), suggesting that even highly expressed EII\textsuperscript{Scr} could be further induced. RT-PCR data also revealed increased transcription of SMU.872 (encoding EII\textsuperscript{Fru}) and decreased transcription of SMU.2047 (encoding EII\textsuperscript{Mal/Glu}) in the presence of sucrose. The EII\textsuperscript{Fru} is a part of the PTS that has been characterized as
a constitutive fructose transporter (11, 41), so it was not surprising to find further increase of transcription of the EII\textsubscript{Fru} gene in sucrose since the fructose moiety of this disaccharide can be released extracellularly. Alternatively, the possibility exists that EII\textsubscript{Fru} might transport sucrose. It was intriguing to find a slight transcriptional decrease of the EII\textsubscript{Mal/Glu} gene. This PTS could also be involved in maltodextrin transport and its lower transcription might be due to a decreased concentration of longer isomaltosaccharides in the culture grown with dextranase.

Several studies have suggested the existence of a second inducible sucrose PTS (26, 33). We did not detect the induction of any PTS, except for those for fructose uptake, following \textit{S. mutans} growth in sucrose. It has been suggested that the second PTS for sucrose was the trehalose PTS in \textit{S. mutans} GS5 (26). We did not detect induction of the trehalose PTS in the presence of sucrose. Furthermore, EII\textsubscript{Tre} exhibited minimal activity under any growth condition except when trehalose was present. Therefore, this PTS cannot be induced by sucrose (or any other sugar tested) in UA159. However, it is possible that this PTS exhibits some affinity for sucrose after it is induced by trehalose. Alternatively, the results presented here may be strain specific since it has been known that there are some strain-specific variations in sugar transport (38).

Similar to sucrose, maltose serves as a primer for the synthesis of water-soluble dextrans (10). These molecules are cleaved by dextranase to isomaltosaccharides that can be taken up by \textit{S. mutans}. Therefore, the maltose/maltotriose ABC transporter (SMU.1568-1571) may also be responsible for transport of longer isomaltosaccharides. RT-PCR data revealed elevated transcription of one PTS (SMU.2047) in the presence of maltose and maltotriose (Fig. 5). This transporter has been previously characterized as a glucose transporter (1, 37). Our transcriptional analysis also suggests its involvement in maltose and maltotriose uptake.
As mentioned earlier, in addition to EII\textsuperscript{Lac} genes, all genes for the lactose-tagatose 6-phosphate pathway were also induced in the presence of lactose. This was an expected result because following lactose uptake, lactose-6-phosphate is cleaved by phospho-\(\beta\)-galactosidase (encoded by the last gene of the operon) to galactose-6-phosphate and glucose (15). Galactose 6-phosphate is then metabolized by the tagatose 6-phosphate pathway. Interestingly, the galactose operon was also induced, suggesting that the galactose moiety of lactose is metabolized simultaneously through the Leloir and tagatose 6-phosphate pathways. The Leloir pathway seems to be the primary pathway for galactose catabolism when \textit{S. mutans} grows on galactose as a sole carbon source. The first enzyme of the Leloir pathway, galactokinase, catalyzes the phosphorylation of galactose to galactose 1-phosphate. Inactivation of the galactokinase gene completely abolished the growth of \textit{S. mutans} in galactose (3, 5). Therefore, the primary galactose uptake might not be mediated by a PTS. However, when galactokinase is intact, both the Leloir and the tagatose 6-phosphate pathways are active following growth in galactose (3). Our microarray data confirmed this result (Table S5). Therefore, \textit{S. mutans} might possess an enzyme that converts some intermediate of the Leloir pathway into one of the intermediates of the tagatose 6-phosphate pathway (and vice versa). Interestingly, transcription of the EII\textsuperscript{Lac} genes was fully induced in galactose, suggesting that this PTS might also transport galactose. If this were true, galactose would be phosphorylated during PTS transport and utilized through the tagatose-6-phosphate pathway. However, strain UA159 cannot grow on galactose if galactokinase is inactivated. Therefore, the lactose PTS might not be a primary transporter for galactose. Although the microarray data (Table S5) clearly showed induction of lactose, tagatose 6-phosphate and galactose operons following growth in galactose, we could not with confidence detect a non-PTS transporter dedicated to galactose transport. This was most likely due to a
different growth rate of UA159 in galactose compared to other sugars in which a large number of genes exhibited differential transcription.

Russell and colleagues have shown that the MSM transporter was responsible for uptake and metabolism of melibiose, raffinose and isomaltotriose and the metabolism of sucrose (29, 35). Our results confirmed that the ABC transporter of the MSM operon is the major transporter for raffinose uptake. Induction of the fructose transporter (Fig. 4B) clearly shows the presence of a fructose moiety released from raffinose by extracellular enzymes, presumably fructan hydrolase (fructanase, exo-β-D-fructosidase) (6, 8, 39) and fructosyltransferase (13). These enzymes are capable of cleaving the fructose moiety from raffinose, which is then efficiently taken up by the cell and utilized as an energy source. The lactose transporter might also be able to take up raffinose or the disaccharide known as melibiose that is left following the release of fructose. Melibiose is cleaved by intracellular alpha-galactosidase to galactose and glucose and these monosaccharides are utilized as an energy source (29). The microarray data clearly show that all three monosaccharides of raffinose and stachyose (galactose, glucose and fructose) are utilized by S. mutans during the mid-log phase of growth and that galactose is metabolized simultaneously through the Leloir and Tagatose 6-phosphate pathway.

In the presence of trehalose, transcription of an operon encoding putative EII\textsuperscript{Tre} (SMU.2038) and trehalose-6-phosphate hydrolase (SMU.2037) was increased. The microarray result confirmed that this operon indeed encodes a major trehalose inducible EII\textsuperscript{Tre} and accompanied hydrolase. Presumably, the PTS takes up and phosphorylates trehalose to trehalose 6-phosphate that is then hydrolyzed to glucose and glucose-6-phosphate. Although the presence of an inducible trehalose PTS was demonstrated previously (26), this is the first study that characterizes the trehalose operon and its transcription.
S. mutans is capable of utilizing β-glucosides such as cellobiose, aesculin, arbutin and salicin. Metabolic and transport genes for these sugars are organized in three loci. Cellobiose and salicin are utilized by proteins encoded in the cel locus (SMU.1596-1601) (22). The second locus, designated bgl (SMU.977-985), consists of the genes for PTS transport (EII, SMU.980) and the metabolism of aesculin (7, 22). Arbutin is hydrolyzed by its own phospho-β-glucosidase (encoded by arb gene; SMU.1102), but it is transported by the cel or bgl systems (22).

Although the microarray data showed that transcription of the EII^cel (SMU.1596, SMU.1598 and SMU.1600) varied several fold in different sugars, the signal was always very low, suggesting that this operon is substrate inducible and there was only a basal level of transcription under all previously tested conditions (Fig. 4). To test this hypothesis, a microarray analysis was performed following growth in cellobiose. As expected, the genes for EII^cel, the accompanying phospho-β-glucosidase (SMU.1601) and the regulator (SMU.1599), were differentially transcribed with an induction level being much higher than the basal level of transcription (Table S11, Fig. 4C). We conclude that cellobiose is transported by an inducible PTS and subsequently hydrolyzed by phospho-β-glucosidase, CelA.

Previous studies have demonstrated that S. mutans possessed two independent inducible PTSs for transport of mannitol and sorbitol (20). The accompanying catabolic enzymes, mannitol-1-phosphate and sorbitol-6-phosphate dehydrogenases that convert the respective phosphorylated sugars to fructose-6-phosphate, were also inducible (20). However, our results showed that mannitol induced both mannitol and sorbitol operons, suggesting that both operons were utilized for transport and metabolism of mannitol in UA159. Consequently, sorbitol EII was renamed EII^Sorb/Mntl.
Analysis of global transcription profiles did not detect significant induction of any sugar transporter in the presence of glucose as compared to any other condition. However, genes for five EIIIs were consistently highly transcribed, and two ABC transporters were moderately expressed, whereas the other transporters showed very low transcription in glucose (Tables S1, S3-S10, Fig. 3A and 4A). The presence of a glucose PTS in S. mutans was reported decades ago (9, 18, 32). In fact, S. mutans harbors at least two glucose PTS transporters (21, 37). One of them has been characterized and demonstrated to take up mannose, glucose and 2-deoxyglucose (2, 37). Another is responsible for glucose, 2-deoxyglucose and α-methylglucoside transport (1, 37).

Comparison of the two glucose PTSs to the genome database (4) showed that they were designated as SMU.1877-1879 (SMU.1877, mannose/glucose-specific EIIAB; SMU.1878, mannose/glucose-specific EIIC; SMU.1879, mannose/glucose-specific EIID) and SMU.2047 (glucose/maltose-specific EIIABC), respectively (Fig. 2). RT-PCR data revealed that the second glucose PTS was also a maltose transporter. Both glucose transporters were consistently highly transcribed in UA159. Additionally, both ABC sugar transporters ABCRaf and ABCMaltd/Mal showed differential transcription in glucose, suggesting they might have some affinity for this carbohydrate.

The five PTS transporters that were highly transcribed in glucose showed a similar level of transcription in all other sugars used in this study (Fig. 3 and 4). It is not completely clear which carbohydrates are substrates for these transporters. As mentioned earlier, EII\text{Mal/Glu} is specific for maltose and glucose, and EII\text{Man/Glu} is specific for mannose and glucose. Sato and colleagues have cloned and sequenced a sucrose-specific transporter of S. mutans (31). Comparison of this sequence to the genome database showed that one of the five highly expressed EII is EII\text{Suc}. In addition to the inducible fructose PTS, S. mutans also possesses a
constitutive fructose PTS (11, 41). Comparison of its sequence to the genome database revealed  
that EII\textsubscript{Fru} was also one of the five highly transcribed transporters. The specificity of the fifth  
highly transcribed EII (SMU.270-272) remains unknown.

Although the five PTSs were highly transcribed under every condition tested in this  
study, some showed transcription that was further elevated if the particular sugar was present.  
The gene for high affinity EII\textsuperscript{Suc}, previously characterized as constitutive, showed further  
increase in transcription in sucrose as compared to glucose (Fig. 5). Similarly, RT-PCR results  
revealed that the transcription of the genes for EII\textsuperscript{Mal/Glu} and EII\textsuperscript{Fru} was elevated several fold in  
the presence of maltose and sucrose, respectively (Fig. 5). This data confirms that the  
transcription of the “constitutive” EII genes can be further increased under the appropriate  
environmental conditions.

Evidence that \textit{S. mutans} constantly expresses five PTSs suggests that their substrates,  
glucose, fructose, maltose and sucrose might be preferable sugars for this organism. These are  
also the main dietary sugars, and therefore this bacterium is capable of their immediate  
utilization as they become available. It is obvious that these transporters are important for \textit{S. mutans} since the cell expresses them continuously. In addition to their role in instant sugar  
uptake, it is also possible that these PTSs are involved in the regulation of sugar transport and in  
catabolite repression. Experiments conducted with EII\textsuperscript{Man/Glu} mutants indicated that preferential  
utilization of glucose over lactose depended on the presence of this EII (37).

Transcription of the genes for PTS Enzyme I and Hpr was consistently high following  
growth in different carbohydrates, suggesting that these genes were not regulated by a particular  
sugar used in this study. EII\textsuperscript{β-glu} (SMU.980) was not induced by any sugar used in this study, but  
it has been previously suggested that it transported a β-glucoside. (7, 22). Two EIIs (SMU.114-
115 and SMU.100-103) were repressed under every condition tested in this study and therefore their sugar specificity remains unknown.

Conclusions

Analysis of the global transcription patterns following growth on thirteen different carbohydrates revealed two types of sugar transporters in *S. mutans*. Members of the PTS family, the primary carbohydrate transporters of Gram-positive bacteria, transported most of the sugars. In fact, monosaccharides, disaccharides, β-glucosides and sugar alcohols were all transported by PTSs. In contrast, the ABC transporters appear to be specialized for transport of oligosaccharides in *S. mutans*.

Differential expression profiles not only identified the sugar transporters, but also confirmed the presence of the previously studied genes involved in the catabolism of sugars and allowed identification of new genes and operons. The microarray results, in conjunction with genome sequencing and annotation, provided information about the exact location and composition of the operons involved in sugar transport and metabolism. In nine operons, transporter genes for EII are an integral part of operons consisting of genes encoding enzymes necessary for the hydrolysis and catabolism of the transported sugar. Colocalization of the genes for sugar transport and catabolism indicates their coregulation. Transcription of the majority of the sugar transporters and accompanied catabolic genes was induced by their specific substrate. The common feature of all of these operons was the presence of a regulatory gene that was presumably responsible for their transcriptional regulation.
Although most of the sugars tested in this study specifically induced genes for their own transport and catabolism, five of the PTSs were consistently highly expressed regardless of the sugar source. These transcription profiles were consistent with the published functional studies for glucose/mannose and fructose PTS. Microarray data provided evidence that maltose/glucose PTS as well as sucrose PTS were also consistently highly expressed, suggesting that glucose, fructose, maltose and sucrose might be the preferred sugars for *S. mutans*. Additionally, these transporters might be involved in the regulation of sugar transport and metabolism, as shown for EII\textsubscript{Man/Glu} (1, 2, 37).

All inducible PTSs showed very low transcription in the absence of the specific sugar substrate, suggesting their high specificity for particular sugars. The ABC transporters showed low to moderate transcription in different sugars, suggesting that they might transport multiple substrates. Interestingly, the transcription of these ABC transporters was very low in sucrose, suggesting their minor role in transport of this carbohydrate.

Our results demonstrate that *S. mutans* possesses inducible transporters for specific sugars and five PTS transporters that are consistently highly transcribed and presumably available for immediate uptake of the common dietary sugars. The capacity of *S. mutans* to rapidly transport and metabolize a wide range of sugars, whenever they become available, may be directly related to its survival in dental plaque and its cariogenic potential in humans.
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References


Figure legends:

**Figure S1 (Supplemental Material).** Growth curves of *S. mutans* UA159 in defined medium supplemented with different sugars. Three to five replicates were generated in each sugar for all experiments. Growth in glucose (A, B, D-L), glucose with dextranase (C), or mannitol (M-N) was compared to growth in: A) fructose; B) mannose; C) sucrose with dextranase; D) lactose; E) galactose; F) raffinose; G) stachyose; H) trehalose; I) maltose; J) maltotriose; K) cellobiose; L) mannitol; M) cellobiose; N) mannose.

**Figure 2. Schematic presentation of the genetic loci involved in transport and catabolism of the sugars analyzed in this study.** Arrows represent the genes. Probable function of each gene is listed below. In some cases function of the gene is redefined as a result of this study as compared to the original annotation. The numbers represent numeric designation of the open reading frames (ORFs) listed in the *S. mutans* UA159 sequencing data base (NCBI) (4). The ORFs are listed in 5’- 3’ order. Description of the gene-products is redefined as compared to the original annotation (4) as a result of this transcriptional study.

- SMU.2047, PTS system, glucose/maltose-specific IIABC;
- SMU.1841, PTS system, sucrose-specific IIABC;
- SMU.1843, sucrose-6-phosphate hydrolase;
- SMU.1844, sucrose operon repressor;
SMU.1185, PTS system, mannitol-specific EIIBC;
SMU.1184, transcriptional regulator, antiterminator;
SMU.1183, PTS system, mannitol-specific EIIA;
SMU.1182, mannitol-1-phosphate dehydrogenase;
SMU.1498, lactose operon repressor;
SMU.1496, galactose-6-phosphate isomerase, subunit LacA;
SMU.1495, galactose-6-phosphate isomerase, subunit LacB;
SMU.1494, tagatose-6-phosphate kinase;
SMU.1493, tagatose-1,6-bisphosphate aldolase;
SMU.1492, PTS system, lactose-specific EIIA (permease EIIA-LAC)
SMU.1491, PTS system, lactose-specific EIIBC (permease EIIBC-LAC)
SMU.1490, 6-phospho-beta-galactosidase;
SMU.1489, part of lactose regulon, LacX
SMU.1488, part of lactose regulon,
SMU.308, sorbitol and mannitol-6-phosphate dehydrogenase;
SMU.309, regulator of sorbitol and mannitol operon;
SMU.310, sorbitol and mannitol operon activator;
SMU.311, PTS system, sorbitol and mannitol -specific EIIC2;
SMU.312; PTS system, sorbitol and mannitol-specific EIIBC;
SMU.313, PTS system, sorbitol and mannitol-specific EIIA;
SMU.314, part of sorbitol and mannitol regulon;
SMU.870, transcriptional regulator of fructose operon;
SMU.871, fructose-phosphate kinase;
SMU.872, PTS system, fructose-specific EIIABC;
SMU.1601, phospho-beta-glucosidase;
SMU.1600, PTS system, cellobiose-specific EIIB;
SMU.1599, transcriptional regulator of cellobiose operon, possible antiterminator;
SMU.1598, PTS system, cellobiose-specific EIIA;
SMU.1597, part of cellobiose operon;
SMU.1596, PTS system, cellobiose-specific EIIC;
SMU.1877, PTS system, mannose/glucose-specific EIIAB;
SMU.1878, PTS system, mannose/glucose-specific EIIC;
SMU.1879, PTS system, mannose/glucose-specific EIID;
SMU.1961, PTS system, mannose/fructose-specific EIIA;
SMU.1960, PTS system, mannose/fructose-specific EIIB;
SMU.1958, PTS system, mannose/fructose-specific EIIC;
SMU.1957, PTS system, mannose/fructose-specific EIID;
SMU.1956, part of the mannose/fructose-specific PTS;
SMU.2040, transcriptional regulator, repressor of the trehalose operon;
SMU.2038, PTS system, trehalose-specific EIIABC;
SMU.2037, trehalose-6-phosphate hydrolase TreA;
SMU.1566, maltodextrin and maltose operon transcriptional repressor;
SMU.1568, maltodextrin and maltose ABC transporter, sugar-binding protein MalX;
SMU.1569, maltodextrin and maltose ABC transporter, MalG permease;
SMU.1570, maltodextrin and maltose ABC transporter, permease;
SMU.1571, ABC transporter, ATP-binding protein;
SMU.876, MSM operon regulatory protein;
SMU.877, MSM operon, alpha-galactosidase;
SMU.878, MSM ABC transporter, sugar-binding protein precursor MsmE;
SMU.879, MSM ABC transporter, permease protein MsmF;
SMU.880, MSM ABC transporter, permease protein MsmG;
SMU.881, MSM operon, sucrose phosphorylase, GtfA;
SMU.882, MSM operon, ABC transporter, ATP-binding protein MsmK;
SMU.883, MSM operon, dextran glucosidase DexB.

**Figure 3.** Differential transcription of the genes for PTS EII and ABC transporters following growth of *S. mutans* UA159 in glucose and fructose. Microarray results are presented as vertical bars. The numerical ORF designation for genes encoding sugar transporters is presented on the X-axis. A full list of the gene-product names is presented in Fig. 2. The 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. A total of ten glucose and ten fructose-grown samples were analyzed in five independent experiments. Two biological replicates were grown and tested in each experiment for each sugar, thus we present the average results for two samples grown under the same condition. Therefore, Conditions: A, glucose; B, fructose.

**Figure 4.** Differential transcription of genes for PTS EII and ABC transporters following growth of *S. mutans* UA159 in different sugars. Microarray results are presented as vertical bars. The numerical ORF designation for genes encoding sugar transporters is presented on the
X-axis. A full list of the gene-product names is presented in Fig. 2. The value of the normalized expression signal is presented on the Y-axis. Two biological replicates were analyzed for each sugar, thus we present the average results for samples grown under each condition. Conditions: A, fructose, mannose, sucrose with dextranase, glucose with dextranase; B, lactose, galactose, raffinose, stachyose; C, trehalose, maltose, maltotriose, cellobiose, mannitol.

Figure 5. Comparison of microarray and RT-PCR results for selected sugar transporter genes. Vertical bars represent fold change of gene transcription of an experimental condition versus baseline. In most cases the baseline was a glucose-grown sample, except for samples grown in cellobiose and mannose that were also compared to mannitol. For both sets of the results, the fold changes are presented in log2 scale (Y-axis). Results presented above the X-axis represent an increase in transcription, and results below the X-axis represent a decrease in transcription. The numerical ORF designation for the sugar transporter genes is presented on the X-axis. A full list of gene-product names is presented in Fig. 2. Experimental conditions (left to right) compared to glucose-grown samples: SMU.1596, cellobiose; SMU.1958 and SMU.1877, mannose; SMU.1185 and SMU.312 mannitol; SMU.878 and SMU.2038 raffinose; SMU.2047, SMU.1568 and SMU.1569 maltose; SMU.1958, SMU.872, SMU.2047, SMU.1841 and SMU.1877 fructose; SMU.2047, SMU.1568 and SMU.1569 maltotriose; SMU.1841, SMU.1568, SMU.1569, SMU.1958, SMU.878, SMU.1877, SMU.2047 and SMU.872 sucrose. Experimental conditions compared to mannitol grown samples: SMU.1596, cellobiose; SMU.1958, mannose.
Figure 5.

Fold Change Presented as Log2

|-----------------------------------------|---------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|