Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions

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Streptococcus mutans often adopts a sessile biofilm lifestyle that differs greatly from that of free-living cells. Biofilm formation represents a protected mode of growth that allows cells to survive in hostile environments. In this study, *in vitro* comparative transcriptome analysis was carried out to identify genes that are differentially expressed in biofilm of *S. mutans* compared with free-living cells. DNA-microarray analyses indicated that about 12 % of genes showed significant differential expression: 139 were activated and 104 were repressed in biofilm vs the planktonic environment. The differential expression of 20 selected genes was confirmed by real-time RT-PCR. In addition, regulation of expression of these genes during biofilm development was tested in 100 and 400 µm deep biofilms. Direct comparison of optical images consistently demonstrated that changes in biofilm thickness are accompanied by significant shifts in cell viability. From evaluation of gene expression patterns, it was shown that the majority of the genes tested were significantly down-regulated in 400 vs 100 µm deep biofilms. This study provides a genome-scale synopsis and adds important insights into gene expression in biofilm development processes of *S. mutans*, which are strongly associated with the pathogenesis of dental diseases.

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INTRODUCTION

Many bacteria in nature are assembled as communities attached to surfaces and form matrix-embedded biofilms (Marsh, 2005). Such biological organization provides a sheltered microenvironment for the immobilized bacteria (Bowden & Hamilton, 1998; Hall-Stoodley *et al.*, 2004). Oral biofilms harbouring pathogenic bacteria are among the major virulence factors associated with dental diseases such as caries and periodontitis (Marsh, 2003; Wilson, 2001).

Streptococcus mutans survives in an extremely diverse, high cell density biofilm on the tooth surface. These bacteria are strongly associated with caries formation (Burne *et al.*, 1997; Hamilton, 2000; Kolenbrander, 2000; Liljemark & Bloomquist, 1996; Munson *et al.*, 2004). Following the adhesion of cells to surfaces and accumulation to form multilayered cell clusters, the biofilm bacteria demonstrate

a radically different phenotype from the planktonic state (Gilmore *et al.*, 2003; Marsh, 2005; Steinberg, 2000). Sessile bacteria are generally much more tolerant to antibiotics, biocides and hydrodynamic shear forces than their planktonic counterparts (Hall-Stoodley *et al.*, 2004; Stewart & Costerton, 2001). These differences in properties between biofilm and planktonic bacteria have led to the hypothesis that there are significant changes in the patterns of gene expression between the two growth modes (Wen & Burne, 2002; Whiteley *et al.*, 2001). We have used DNA microarray and quantitative RT-PCR technologies to identify transcriptional changes that accompany the formation of this persistent physiological state of *S. mutans*. We also characterized the physiological and genetic differences of this bacterium, in biofilms of various depths.

METHODS

Bacterial strains and culture conditions. *S. mutans* UA159 was grown for 24 h in brain heart infusion broth (BHI, Difco Labs) at 37 °C in 95 % air/5 % CO₂ (v/v). Cultures of *S. mutans* were diluted 1:50, inoculated into fresh BHI medium and grown in polystyrene test tubes for an additional 24 h (37 °C, 95 % air/5 % CO₂ (v/v), final pH 5.0 ± 0.2). For biofilm generation (Senadheera *et al.*, 2005), 20 µl of *S. mutans* culture grown as described above was seeded in 20 mm diameter, 15 mm deep sterile polystyrene multidishes (NUNC-143982; Nunc). After 24 h, the biofilm (final pH 5.1 ± 0.1) was washed with 1 ml ice cold sodium chloride (9 g l⁻¹).

Abbreviations: BHI, brain heart infusion; CDFF, constant-depth film fermenter; CLSM, confocal laser scanning microscope; PTFE, polytetrafluoroethylene; TIGR, The Institute for Genomic Research.

The GEO Series accession number for the microarray data discussed in this paper is GSE6744.

The online version of this paper (at <http://mic.sgmjournals.org>) includes supplementary tables listing the genes displaying differences in expression in biofilms vs planktonic cultures.

RNA extraction. Extraction of total RNA was performed as described previously (Tam *et al.*, 2006) with slight modifications. Biofilm-grown *S. mutans* cells were suspended in TRI Reagent (Sigma-Aldrich) and dislodged by scraping into a 2 ml microcentrifuge tube containing 0.4 ml of 1 mm diameter glass beads (Sigma-Aldrich). The cells were disrupted with the aid of a Fast Prep Cell Disrupter (Bio 101, Savant Instruments), centrifuged and the RNA-containing supernatant was supplemented with 1-bromo-3-chloropropane (BCP; Molecular Research Center). The upper aqueous phase was precipitated with 2-propanol. After centrifugation, the resulting RNA pellet was washed with ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Because of the sensitivity of the PCR, residual contaminating DNA was eliminated by incubation of the sample with RNase-free DNase (Promega). The DNase was then inactivated by incubation at 65 °C for 10 min, and the RNA was precipitated with ethanol and resuspended in DEPC-treated water. The RNA concentration was determined spectrophotometrically using the Nanodrop instrument (ND-1000, Nanodrop Technologies). The integrity of the RNA was determined by agarose gel electrophoresis (data not shown). The same procedure was conducted for RNA extraction from planktonic cells, which were collected in stationary phase by centrifugation (4500 g, 4 °C) and immediately resuspended in TRI Reagent (Sigma-Aldrich) followed by the same protocol as described above for biofilm bacteria. The biofilms and planktonically grown cell pellets were stored at -20 °C until RNA isolation.

Microarray design, cDNA labelling and hybridization. Microarray slides were obtained from the Pathogen Functional Genomics Resource Center (PFGR) located at The Institute for Genomic Research (TIGR). The arrays consisted of 1948 70-mer oligonucleotides representing 1960 ORFs from *S. mutans* UA159 and additional control sequences. The full 70-mer complement was printed four times on the surface of each microarray slide. Details of the microarrays and probe labelling, hybridization and washing conditions can be found at <http://www.tigr.org>. Synthesis of cDNA and labelling was performed using a method similar to that described in <http://pfgrc.tigr.org/protocols/M007.pdf> as follows. cDNA was generated with random hexamers (6 µg) from 2 µg total RNA using the superscript III reverse transcriptase (Invitrogen). The reverse transcription reaction was carried out at 42 °C overnight with aminoallyl (aa) labelled dUTP. Removal of unincorporated aa-dUTP and free amines was carried out using Microcon YM-30 (Millipore) filters according to the manufacturer's recommendations. Coupling of aminoallyl labelled cDNA to Cy-dye esters was performed in 0.1 M sodium carbonate buffer (pH 8.6) for 1 h at room temperature. Removal of free dyes was accomplished with Qiagen QIAquick PCR purification columns. The labelling level was quantified using a spectrophotometer (ND-100, Nanodrop Technologies). Each Cy3-labelled sample was mixed with an equal amount of Cy5-labelled sample, and the mixtures were allowed to dry in a speed vacuum for about 1 h. Hybridization was performed basically according to TIGR protocol (<http://pfgrc.tigr.org/protocols/M008.pdf>). The samples were resuspended in hybridization buffer containing 50 % formamide, 5 × SSC, 0.1 % SDS and 20 µg yeast tRNA, and heated at 95 °C for 5 min. Following a quick centrifugation, the Cy dye-coupled probes were applied to microarray slides and incubated overnight at 42 °C. Hybridization was carried out in Corning hybridization chambers submerged in a water bath. The hybridized slides were then washed and scanned by using an Axon GenePix 4000B Scanner (Axon Instruments) with settings adjusted in order to obtain a similar green and red overall intensity.

Array data normalization and statistical analysis. The results represent the findings from three independent biological replicate arrays performed with three different RNA samples. In two of the arrays the biofilm RNA sample was labelled with Cy5 and the

planktonic sample with Cy3, while the third array was labelled with the reverse combination of samples and dyes. The hybridization patterns and intensities were quantitatively analysed using the GenePix Pro 4.1 software (Axon) on scanned microarray images. This software localizes every spot within a programmed grid to obtain a mean signal density of each spot and background (offset) of the area surrounding the spot. The resulting files (gpr) produced by GenePix were analysed utilizing the LIMMA (Smyth, 2004) software package, available from the CRAN site (<http://www.r-project.org>). Spots flagged as not found or absent in GenePix were removed by filtering. Another filter was applied for saturated spots. After filtering, the data within the same slide were normalized using global loess normalization with the default smoothing span of 0.3 (Smyth & Speed, 2003). To identify differentially expressed genes, a parametric empirical Bayesian approach implemented in LIMMA was used (Lonnstedt & Speed, 2002). A moderated *t*-test was performed in parallel, with the use of a false discovery rate (Reiner *et al.*, 2003) correction for multiple testing. TIGR arrays include four replicates for each gene. Instead of just taking the mean of replicate spots, we used the duplicate correlation function (Smyth *et al.*, 2005) available in LIMMA to acquire an approximation of gene-by-gene variance. This method greatly improves the precision with which the gene-wise variances are estimated and thereby maximizes inference methods designed to identify differentially expressed genes. A *P* value <0.05 confidence level was used to pinpoint those significantly differentiated genes. Genes had to have an *A*-value (mean expression level for the gene across all arrays and channels) of more than 7.5, leaving out genes with a mean intensity in both channels of less than 200.

CDFF and confocal laser scanning microscopy. The regulation of gene expression during biofilm development was tested in a constant-depth film fermenter (CDFF). Biofilms were grown by a method similar to that previously described (Pratten & Wilson, 1999). The CDFF consisted of a rotating turntable with adjusted polytetrafluoroethylene (PTFE) cylinders on which the biofilms were grown at depths of 100 or 400 µm, as follows. Pure cultures of *S. mutans* UA159 were cultivated overnight at 37 °C in 700 ml BHI. Next, the inoculum was pumped into the CDFF for 7 h via a peristaltic pump (ISMATEC SA) at a rate of 100 ml h⁻¹ at 37 °C. BHI was delivered into the CDFF at a rate of 100 ml h⁻¹. After 72 h at 37 °C, the PTFE cylinders were removed from the CDFF, washed gently with sterile PBS, and bacteria were harvested and subjected to RNA extraction as described above. The biofilms developed on other plugs were stained with LIVE/DEAD *BacLight* fluorescent dye (Molecular Probes) for 10 min. Fluorescence emission of the PBS-washed samples was measured using a Zeiss LSM 510 (Carl Zeiss Microscopy) confocal laser scanning microscope (CLSM). In each experiment, exciting laser intensity, background level, contrast and electronic zoom size were maintained at the same level. At least three random fields were analysed in each experiment. A series of optical cross-section images were acquired at 5 µm depth intervals from the surface through the vertical axis of the specimen, using a computer-controlled motor drive. 3D confocal images were reconstituted and processed for display using Adobe Photoshop 7.0 software.

Reverse transcription and real-time quantitative PCR. The array hybridization data were confirmed for a subset of 20 genes/ORFs (see Results) by quantitative SYBR green PCR assays employing an ABI-Prism 7000 LightCycler System (Applied Biosystems). The RT-PCR reaction, on the same RNA samples as generated for microarray experiments, was performed as described previously (Shemesh *et al.*, 2006). The corresponding oligonucleotides primers were designed using the algorithms provided by Primer Express (Applied Biosystems) for uniformity in size (≈90 bp) and melting temperature. For each set of primers, a standard amplification curve was plotted (critical threshold cycle against log of concentration)

and only those with slope ≈ -3 were considered reliable primers. A reverse transcription (RT) reaction mixture (20 μ l) containing 50 ng random hexamers, 10 mM dNTPs mix and 1 μ g total RNA sample was incubated at 65 °C for 5 min to remove any secondary structure, and placed on ice. Then 10 $\times$ RT buffer, 25 mM MgCl₂, 0.1 M DTT, 40 U RNaseOUT recombinant ribonuclease inhibitor and 50 U Super Script II RT (Invitrogen) were added to each reaction mix. After incubation at 25 °C for 10 min, the mixture was incubated at 42 °C for 50 min. The reaction was terminated by heating the mixture at 70 °C for 15 min, and the cDNA samples were stored at -20 °C until used.

The RT-PCR reaction mixture (20 μ l) contained 1 $\times$ SYBR Green PCR Master Mix (Applied Biosystems), 1 μ l cDNA sample and 0.5 μ M of the appropriate forward and reverse PCR primers. PCR conditions included an initial denaturation at 95 °C for 10 min, followed by a 40-cycle amplification consisting of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. All primer pairs were checked for primer dimer formation by using the two-step protocol described above without the addition of RNA template. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out in the absence of reverse transcriptase in order to identify whether the RNA samples were contaminated by residual genomic DNA. The critical threshold cycle (C_t) was defined as the cycle in which fluorescence becomes detectable above the background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with C_t values obtained from amplification of known quantities of *S. mutans* cDNA. The standard curves were used for transformation of the C_t values to the relative number of cDNA molecules. The contamination of genomic DNA was determined from control reactions, devoid of reverse transcriptase. The same procedure was repeated for all the primers.

The expression levels of all the genes tested by real-time RT-PCR (see Table 2) were normalized using the 16S rRNA gene of *S. mutans* (GenBank accession no. X58303) as an internal standard. There was no significant difference in the expression of the 16S rRNA gene in the various conditions and samples tested (data not shown). Each assay was performed with at least two independent RNA samples in duplicate and the x -fold change of the transcription level was calculated by the following equations (ABI Prism 7000 SDS Software v1.1 with RQ Study 1.0, Applied Biosystems):

$$\text{For each cDNA: } \Delta C_t = C_{t(\text{target gene})} - C_{t(16S \text{ rRNA})}$$

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

$$\text{Ratio} = 2^{-\Delta\Delta C_t}$$

Statistical analysis for real-time RT-PCR data. Student's t -test was used to calculate the significance of the difference between the mean expression of a given gene in a biofilm and its mean expression in planktonic culture. The same test was used to analyse the differences in gene expression between 100 and 400 μ m deep biofilms. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Biofilm formation can be considered a developmental process, which is characterized through the identification of structural and regulatory genes required at the various steps of the process (O'Toole *et al.*, 2000; Steinberg, 2000). To gain an insight into similarities and differences in global gene expression between growth in planktonic and biofilm environments, we used *S. mutans* DNA microarray analysis.

We employed this technique for a comparative transcriptome analysis of global gene expression under biofilm and planktonic growth conditions. Relatively few of these genes showed significant differential expression in biofilms compared with planktonic cultures (see Supplementary Table S1, available with the online version of this paper). These results are in agreement with those from a previous study (Whiteley *et al.*, 2001), which showed no dramatic differences in the patterns of gene expression in *Pseudomonas aeruginosa* biofilms, despite the striking differences in their phenotype. It was noticeable that the differences in expression levels of *S. mutans* genes were small compared to those of highly regulated genes in other eubacteria, meaning that the transition to biofilm from the planktonic condition does not radically alter the transcriptome. However, closer assessment reveals that those changes in gene expression have the potential to profoundly affect cellular physiology, which adapts the bacteria to the biofilm environment.

An empirical Bayesian method (B -test) was applied to test for differential expression between planktonic and biofilm environments. Analysis of microarray images revealed a total of 243 genes which were differentially regulated at confidence level of $P < 0.05$ (Supplementary Table S2). Of these genes, 139 were up-regulated and 104 were down-regulated. Significance scores and degrees of differential expression are listed in Table 1 for the 70 most differentially regulated genes (i.e., fulfilling the criteria $B > 0$ in the B -test, more than a 1.6-fold change and $P < 0.005$ in the moderated t -test), in biofilm compared to planktonic conditions. Of these genes, 43 were up-regulated and 27 down-regulated. Almost half of the up-regulated genes encode hypothetical proteins of as yet unknown function present in the *S. mutans* genome (Table S2).

Numerous genes, among the up-regulated ones, are most likely involved in intracellular trafficking and/or membrane transporter systems, such as SMU.1598, SMU.1135, SMU.997 and SMU.998. Genes SMU.1135, SMU.997 and SMU.998 encode a putative ABC transporter: ATP-binding and periplasmic ferrichrome-binding proteins respectively. The ABC transporter superfamily is responsible for the translocation of a wide variety of substances into or out of cells. These proteins exhibit specificity for amino acids, carbohydrates, oligopeptides, osmoprotectants, inorganic ions, bacteriocins and DNA (Ajdic *et al.*, 2002; Davidson & Chen, 2004). However, a substantial number of the up-regulated genes are likely to be involved in transcription and energy metabolism, such as SMU.881, SMU.1539 and SMU.105. The genes SMU.1905c, SMU.1714c and SMU.1914c encode proteins which are probably involved in the biosynthesis of bacteriocins. *S. mutans* possesses the ability to produce these naturally occurring antibiotic peptides, which enable it to persist in the oral biofilm community (Merritt *et al.*, 2005).

Regulatory genes were found to have differential effects on biofilm formation and are also important virulence factors

Table 1. The most significant ($P < 0.005$) differentially expressed genes of *S. mutans* in biofilms vs planktonic cells according to B-value

Genes are listed according to their decreasing statistical importance.

Locus no.*	log ₂ ratio	Fold change†	Description*	t‡	P‡	B§
SMU.237c	-2.46	5.51	Putative integral membrane protein	-11.18	3.51E-05	9.85
SMU.1360c	3.35	10.21	Hypothetical protein	14.07	4.39E-05	8.74
SMU.1908c	2.33	5.03	Hypothetical protein	9.94	4.99E-05	8.41
SMU.1014	1.99	3.97	Hypothetical protein	9.75	4.99E-05	8.18
SMU.494	1.56	2.95	Putative transaldolase	9.98	1.12E-04	7.27
SMU.881	1.13	2.19	Sucrose phosphorylase, GtfA	8.83	1.15E-04	6.97
SMU.1914c	1.73	3.32	Hypothetical protein	8.61	1.34E-04	6.67
SMU.105	1.78	3.44	Putative transcriptional regulator repressor of sugar transport	8.83	1.48E-04	6.49
SMU.1912c	3.98	15.77	Hypothetical protein	10.32	1.79E-04	6.17
SMU.1421	1.11	2.16	Putative dihydrolipoamide acetyltransferase, E2 component	8.15	1.79E-04	6.03
SMU.1039c	2.37	5.17	Putative lipopolysaccharide glycosyltransferase	9.90	2.36E-04	5.82
SMU.150	1.22	2.33	Hypothetical protein	7.80	2.53E-04	5.51
SMU.233	-1.29	2.44	Ketol-acid reductoisomerase	-7.65	2.93E-04	5.28
SMU.2032	-1.15	2.22	30S ribosomal protein S2	-7.58	3.01E-04	5.18
SMU.2074	-1.91	3.76	Putative anaerobic ribonucleoside-triphosphate reductase	-7.48	3.11E-04	5.02
SMU.1861c	1.57	2.97	Hypothetical protein	7.48	3.11E-04	5.01
SMU.1909c	2.62	6.13	Hypothetical protein	7.33	3.49E-04	4.79
SMU.1539	1.47	2.77	Putative 1,4- α -glucan branching enzyme	7.32	3.49E-04	4.77
SMU.493	1.44	2.71	Formate acetyltransferase (pyruvate formate-lyase 2)	7.58	3.49E-04	4.78
SMU.805c	-1.41	2.66	Putative amino acid ABC transporter, ATP-binding protein	-7.25	3.53E-04	4.66
SMU.1135	1.19	2.27	Putative phosphate ABC transporter, ATP-binding protein	7.10	4.24E-04	4.43
SMU.1906c	1.52	2.86	Hypothetical protein	7.06	4.34E-04	4.36
SMU.2024c	-0.96	1.95	50S ribosomal protein L4	-6.92	5.08E-04	4.13
SMU.124	1.13	2.19	Putative transcriptional regulator (MarR family)	7.16	5.08E-04	4.18
SMU.1424	1.62	3.08	Putative dihydrolipoamide dehydrogenase	7.42	5.26E-04	4.16
SMU.1586	-1.11	2.17	Putative threonyl-tRNA synthetase	-6.81	5.39E-04	3.96
SMU.58	2.16	4.47	Hypothetical protein	8.22	5.39E-04	4.20
SMU.1423	1.18	2.27	Putative pyruvate dehydrogenase, TPP-dependent E1 component	6.72	5.95E-04	3.81
SMU.1573	-1.16	2.23	Putative S-adenosylmethionine synthetase	-6.51	8.14E-04	3.46
SMU.104	2.25	4.77	Putative α -glucosidase glycosyl hydrolase	6.30	1.10E-03	3.10
SMU.224c	1.69	3.22	Hypothetical protein	7.47	1.10E-03	3.38
SMU.1538	1.25	2.37	Putative glucose-1-phosphate adenylyltransferase ADP-glucose	7.41	1.15E-03	3.28
SMU.508	1.03	2.04	Conserved hypothetical protein	6.22	1.16E-03	2.97
SMU.1346	1.98	3.94	Putative thioesterase bact	7.31	1.21E-03	3.20
SMU.225c	1.66	3.15	Hypothetical protein	7.64	1.43E-03	3.08
SMU.883	0.83	1.77	Dextran glucosidase DexB	5.94	1.71E-03	2.47
SMU.1173	0.85	1.80	Putative O-acetylhomoserine thiolase	5.93	1.71E-03	2.46
SMU.960	-1.02	2.02	50S ribosomal protein L7/L12	-5.92	1.71E-03	2.44
SMU.2065	-1.26	2.39	Putative UDP-glucose 4-epimerase	-5.87	1.81E-03	2.36
SMU.813	1.98	3.95	Hypothetical protein, putative transcriptional regulator	6.80	1.93E-03	2.58
SMU.2031	-1.05	2.07	Putative translation elongation factor TS	-5.79	1.93E-03	2.22
SMU.1598	2.13	4.38	Putative PTS system, cellobiose-specific IIA component	5.96	1.93E-03	2.27
SMU.780	0.77	1.70	Putative chorismate synthase	5.64	2.47E-03	1.95
SMU.1493	1.01	2.02	Tagatose-1,6-bisphosphate aldolase	5.77	2.55E-03	1.95
SMU.1022	2.12	4.34	Conserved hypothetical protein, CitG homologue	7.42	2.58E-03	2.32
SMU.1182	-0.87	1.82	Mannitol-1-phosphate dehydrogenase	-5.54	2.79E-03	1.76
SMU.1203	-0.70	1.63	Putative branched-chain amino acid aminotransferase IlvE	-5.44	3.23E-03	1.57
SMU.1322	-1.00	2.00	Putative acetoin dehydrogenase	-5.43	3.23E-03	1.57
SMU.159	-1.07	2.10	Conserved hypothetical protein	-5.53	3.43E-03	1.54
SMU.1247	-0.91	1.88	Conserved hypothetical protein	-5.35	3.46E-03	1.42
SMU.814	1.45	2.73	Putative MutT-like protein	6.14	3.46E-03	1.74

Table 1. cont.

Locus no.*	log ₂ ratio	Fold change†	Description*	t‡	P‡	B§
SMU.129	1.17	2.25	Putative dihydrolipoamide acetyltransferase	5.49	3.46E-03	1.46
SMU.461	-0.92	1.90	Putative amino acid ABC transporter, ATP-binding protein	-5.34	3.46E-03	1.40
SMU.1820c	-1.32	2.50	Putative glutamyl-tRNA ^{Gln} amidotransferase A subunit	-5.25	4.04E-03	1.23
SMU.1910c	2.52	5.75	Hypothetical protein	5.22	4.13E-03	1.17
SMU.2021	-0.77	1.71	30S ribosomal protein S3	-5.34	4.13E-03	1.19
SMU.588	1.70	3.25	Conserved hypothetical protein	5.95	4.13E-03	1.47
SMU.1038c	1.67	3.18	Putative response regulator	5.19	4.13E-03	1.12
SMU.668c	-1.08	2.12	Ribonucleotide reductase, large subunit	-5.19	4.13E-03	1.12
SMU.51	-1.00	2.00	Putative phosphoribosylaminoimidazole carboxylase, ATPase	-5.31	4.14E-03	1.15
SMU.103	1.94	3.84	Putative PTS system, IIA component	5.29	4.22E-03	1.11
SMU.59	-1.03	2.04	Adenylosuccinate lyase	-5.15	4.22E-03	1.04
SMU.137	-0.69	1.61	Malolactic enzyme	-5.11	4.47E-03	0.97
SMU.1096	-0.92	1.89	Putative ABC transporter, ATP-binding protein, choline transporter	-5.09	4.51E-03	0.93
SMU.155	-0.81	1.75	Polyribonucleotide nucleotidyltransferase	-5.09	4.51E-03	0.93
SMU.756	0.96	1.94	Conserved hypothetical protein	5.07	4.56E-03	0.90
SMU.1528	-1.24	2.36	F ₀ F ₁ membrane-bound proton-translocating ATPase, β subunit	-5.06	4.62E-03	0.88
SMU.1037c	2.17	4.50	Putative histidine kinase	5.72	4.77E-03	1.16
SMU.1226c	0.70	1.62	Conserved hypothetical protein	5.01	4.90E-03	0.79
SMU.359	-1.13	2.19	Translation elongation factor G (EF-G)	-4.98	5.10E-03	0.73

*Based on the genome annotation of *S. mutans* provided by TIGR.

†Fold expression change according to the *M* value (log₂ ratio), *M*>0 means up-regulation and *M*<0 down-regulation of the gene.

‡Moderated *t*-test and its corresponding *P* value, adjusted by the Benjamini and Yekutieli method.

§Bayesian test value, which corresponds to the probability of a gene really being differentially expressed.

(Graham *et al.*, 2002; Henke & Bassler, 2004; Senadheera *et al.*, 2005; Yoshida & Kuramitsu, 2002; Zhang *et al.*, 2004). SMU.1037c encodes a putative histidine kinase (Fig. 1), two-component system that shows some similarity to the *vicRK* signal transduction system. The *vicRKX* operon is essential for the viability of *S. mutans*. *vic* gene products appear to modulate adherence, biofilm formation and genetic competence development in *S. mutans* (Biswas & Biswas, 2006). The *vicRKX* operon regulates the expression of several virulence-associated genes affecting synthesis of polysaccharides, including *gtfBCD*, *ftf*, and polysaccharide-binding sites as *gfbpB* (Senadheera *et al.*, 2005). Computational comparisons indicate that the SMU.1037c-encoded protein is highly homologous to a family of autophosphorylating histidine protein kinases (HPKs). Phosphotransfer-mediated signalling pathways allow cells to sense and respond to environmental stimuli (Stock *et al.*, 2000). Consistent with a possible regulatory role in biofilm formation, SMU.1037c is significantly up-regulated in biofilm (Tables 1 and 3).

Other up-regulated regulatory genes include SMU.629 and SMU.1591, encoding homologues of the manganese-type superoxide dismutase (SOD) and catabolite control protein A (CcpA), respectively. In streptococci SOD plays an important role in oxidative stress tolerance (Wen *et al.*, 2006). CcpA protein (designated also as RegM) is highly required for optimal *ftf* and *gtf* expression in *S. mutans* (Brownhardt *et al.*, 2004).

Most of the down-regulated genes are most likely to be involved in protein synthesis or to encode membrane proteins/transporters, such as SMU.237c, SMU.2032, SMU.2024c and SMU.1586 (Table 1). This reduced level of expression may indicate a limited bacterial growth rate and/or that the *S. mutans* organisms in biofilm environments have limited but more specific metabolic activity.

Among the down-regulated genes was SMU.2028, encoding fructosyltransferase (FTF). FTF synthesizes fructan polymers from sucrose (Shemesh & Steinberg, 2006), which are considered to be extracellular storage compounds and can also act as binding sites for bacterial accumulation (Burne *et al.*, 1996; Rozen *et al.*, 2001). The ability of *S. mutans* to produce extracellular fructans plays a role in sucrose-dependent bacterial adherence and biofilm accumulation (Steinberg, 2000). From the point of view of bacteria harbouring the biofilm, it makes bio-economic sense to down-regulate this sucrose-dependent cell-cell adhesion and biofilm formation gene, since sucrose is absent in the environment.

The F₁F₀ membrane-bound proton-translocating ATPase, α and β subunits (SMU.1530 and SMU.1528 respectively), were also found to be down-regulated in biofilm. The F₁F₀-ATPase is largely responsible for the maintenance of homeostatic pH levels in *S. mutans* (Quivey *et al.*, 2000; Wen *et al.*, 2006). The genes for the F₁F₀-ATPase are arranged as an operon (Ajdic *et al.*, 2002). The

Table 2. Nucleotide sequences of primers for genes whose expression in biofilm and planktonic cultures was compared

ORF*	Gene description	Primer sequences (5'–3')	
		Forward	Reverse
16S rRNA	Normalizing internal standard	CCTACGGGAGGCAGCAGTAG	CAACAGAGCTTTACGATCCGAAA
SMU.103	Mannose-specific PTS system, IIA component (<i>sorA</i>)	CCATAGAATTTCTAGCTGGTAT	TCTCCAATCGGTTTCCCATC
SMU.104	α -Glucosidase glycosyl hydrolase	GCAGGGATTCGTGCATTG	AATCGAAGGTTGCAGGCTCTT
SMU.105	Putative transcriptional regulator repressor of sugar transport	TTCCCAATGCTAGAGCTCGG	AAGGACTCCACGTCGTTGGT
SMU.237c	Integral membrane protein	CGCCAATTCTTATGGGCTTC	CCTGTCGTGCGCTCCTTAA
SMU.494	Putative transaldolase	TCATGCACAAGTGTTGCG	AGATTGCCGCCAAGTTCATC
SMU.526c	Transcriptional regulator of multidrug-efflux transporter	CATTAATGAACGACGCCAGTGT	AAAGGCACCTGCTTGATAG
SMU.881	Sucrose phosphorylase (<i>gtfA</i>)	AGGAAGTGAAGCGGCCAGT	TCAATACGGCCATCCAAATCA
SMU.910	Glucosyltransferase-I (<i>gtfD</i>)	ACAGCAGACAGCAGCCAAGA	ACTGGGTTTGCTGCGTTTG
SMU.997	Putative inorganic ion ABC transporter, ATP-binding protein	AGCGCTTGCTTATATGGAAC TTG	CTCGTTGTAATTGACCGCCA
SMU.1004	Glucosyltransferase B (<i>gtfB</i>)	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
SMU.1005	Glucosyltransferase-SI (<i>gtfC</i>)	GGTTTAACGTCAAATTAGCTGTATTAGC	CTCAACCAACCGCCACTGTT
SMU.1037c	Putative histidine kinase	TTCGAACACCCTTGACCATTG	CCAAATCGGCTGCATCAACT
SMU.1039c	Lipopolysaccharide glycosyltransferase	TGCCGGCAGAAGATTAAACC	TGAGAACGTCTTGATCCGGTAA
SMU.1494c	Tagatose-6-phosphate kinase	TGAAACGCATAAAACACCCG	AGTTTCCCACCAAGAAGGCC
SMU.1517	Histidine kinase two-component regulatory system (<i>vicR</i>)	TGACACGATTACAGCCTTTGATG	CGTCTAGTTCTGGTAACATTAAGTCCAATA
SMU.1910c	Hypothetical protein	TGTCTACTGGCCGAATGGATT	CAGATGCGAGCCGCTTTAAC
SMU.1912c	Hypothetical protein	TTGGACAAAGAAAAC TTCATCCT	CATGCTTTTCTATGCGGTCTATTG
SMU.1916	Competence-stimulating system (<i>comDE</i>)	ACAATTCCTTGAGTTCCATCCAAG	TGGTCTGCTGCCTGTTGC
SMU.2028	Fructosyltransferase (<i>ftf</i>)	AAATATGAAGGCGGCTACAACG	CTTCACCAGTCTTAGCATCCTGAA
SMU.2044	Guanosine tetra (penta)-phosphate synthetase (<i>relA</i>)	ACAAAAAGGGTATCGTCCGTACAT	AATCACGCTTGGTATTGCTAATTG

*Based on the genome annotation of *S. mutans* provided by TIGR.

Fig. 1. Schematic diagram of the most highly up-regulated gene loci and their flanking regions. SMU.1037c encodes a putative histidine kinase; SMU.1038c and SMU.1039c encode a putative response regulator and a putative lipopolysaccharide glycosyltransferase, respectively. SMU.99–SMU.103 encode a putative PTS system transport and binding proteins; SMU.104 encodes α -glucosidase glycosyl hydrolase and SMU.105 encodes a repressor for transcriptional regulator of sugar transport. SMU.1908c–SMU.1912c encode hypothetical proteins; SMU.1913c encodes a BlpL-like immunity protein and SMU.1914c encodes a BlpO-like protein. Arrows represent the orientation of each ORF, and the numbers underneath indicate their respective positions on the chromosome, according to the NCBI *S. mutans* genome database.

Table 3. Comparison of real-time RT-PCR and microarray expression values of selected *S. mutans* genes

Locus number*	Putative identification*	Fold difference (biofilm vs planktonic cells)	
		Microarray (<i>P</i> value)	RT-PCR†
SMU.103	Mannose-specific PTS system, IIA component	3.84 (0.004215)	10.18 ± 5.08‡
SMU.104	Putative α -glucosidase glycosyl hydrolase	4.77 (1.10E–03)	6.85 ± 2.45‡
SMU.105	Putative transcriptional regulator repressor of sugar transport	3.44 (1.48E–04)	6.3 ± 3.1‡
SMU.237c	Putative integral membrane protein	–5.51 (3.51E–05)	–9.34 ± 2.15‡
SMU.494	Putative transaldolase	2.95 (0.000112)	6.6 ± 2.4‡
SMU.526c	Putative transcriptional regulator	2.36 (0.037847)	13.1 ± 8.9‡
SMU.881	Sucrose phosphorylase, <i>gtfA</i>	2.19 (0.000115)	8.75 ± 1.4‡
SMU.910	Glucosyltransferase I, <i>gtfD</i>	–1.77 (0.011354)	–1.16 ± 0.01‡
SMU.997	Putative inorganic ion ABC transporter, ATP-binding protein	4.3 (0.017152)	6.16 ± 3.54
SMU.1004	Glucosyltransferase B, <i>gtfB</i>	1.05 (0.9)	–3.22 ± 2.04
SMU.1005	Glucosyltransferase-SI, <i>gtfC</i>	1.45 (0.14)	2.95 ± 0.75‡
SMU.1037c	Putative histidine kinase	4.5 (0.004772)	9.5 ± 5.5‡
SMU.1039c	Putative lipopolysaccharide glycosyltransferase	5.17 (0.000236)	13.15 ± 8.35‡
SMU.1494c	Tagatose-6-phosphate kinase	1.79 (0.04)	15.23 ± 8.7
SMU.1517	Histidine kinase, <i>vicR</i>	1.5 (0.22)	2.79 ± 0.72‡
SMU.1910c	Hypothetical protein	5.75 (0.004125)	10.23 ± 7.7
SMU.1912c	Hypothetical protein	15.77 (0.000179)	30.65 ± 8.35‡
SMU.1916	Two-component signal transduction system, <i>comDE</i>	1.31 (0.52)	4.56 ± 1.66‡
SMU.2028	Levansucrase (β -D-fructofuranosyl transferase), <i>ftf</i>	–1.57 (0.021065)	1.05 ± 0.25
SMU.2044	Guanosine tetra (penta)-phosphate synthetase, <i>relA</i>	1.03 (0.92)	1.72 ± 0.19*

*Locus number and putative identification are according to TIGR *S. mutans* genome database.

†Expression values are expressed as mean experimental starting quantity/mean of control starting quantity. Data show mean ± SD from at least two independent experiments.

‡Comparisons of means were significantly different ($P < 0.05$).

Fig. 2. Correlation of gene expression data obtained by DNA microarray and real-time RT-PCR in biofilms compared with planktonic cultures. Points on the graph represent genes that were analysed by both methods. Fold changes in transcript abundances after comparison of biofilm and planktonic growth phases are plotted; the line indicates the linear regression ($R^2 = 0.8704$).

down-regulation of F_1F_0 -ATPase appears to indicate that the bacteria are much less exposed to pH changes in biofilms.

The validity of the above microarray data was examined using real-time RT-PCR for differential expression analysis of selected genes (Table 2). The genes for this analysis were selected as being either highly up-regulated or highly down-regulated, associated with virulence and of known function rather than hypothetical. When expression of this panel of genes was assessed by real-time RT-PCR in planktonic culture and biofilm, the ratios of expression correlated with the ratios obtained by microarray analyses (Table 3) with a correlation coefficient (R^2)-value of 0.87 (Fig. 2). The most prominent differences between the array and RT-PCR approaches comprised the set of non-expressed and low-level-expressed genes, which was probably due to the inherent technical variability of the microarray technique. Another reason for the residual variation between the two techniques could be associated with the incorporation of labelling compounds only for the microarray technique and the intrinsic dependence on the enzyme used for labelling (Bustin, 2000). Overall, the observed correlation between the results obtained by the two techniques was positive.

One of the essential factors in mature biofilm development is regulation of the formed biofilm. The biofilm microenvironment is affected by limits of influx and efflux of nutritional and bacterial metabolites, quorum sensing, density and changes in gas tension (Steinberg 2000; Bowden & Li, 1997). As biofilm thickness plays a crucial role in these experimental factors, we conducted this study under controlled nutrient flow and controlled biofilm depth conditions, by using a constant depth film fermenter (CDFF). A visible film of adherent cells was observable on the PTFE cylinders 24 h after inoculation. The biofilm continued to develop and eventually formed confluent biofilms of 100 or 400 μ m depth, which were further

assessed by CLSM (Fig. 3), in an attempt to evaluate the viability of the biofilm populations. Direct comparison of the optical images consistently demonstrated that biofilm thickness is accompanied by significant shifts in cell physiology. As estimated by CLSM, bacteria grown at 100 μ m depth (Fig. 3a) showed more vitality than those cultured at 400 μ m (Fig. 3b). This decrease in viability might be due to restriction in nutrient availability when the biofilm is thicker.

Evaluating gene expression patterns in different biofilm depths, by real-time RT-PCR, revealed that the majority of the genes tested were significantly down-regulated in 400 μ m vs 100 μ m deep biofilms (Fig. 4). A possible explanation for these results could be that the selected genes are activated mostly in the initial step of biofilm development. Those genes might be responsible for switching between a sessile/adhesive and motile/planktonic cell phenotype and not necessarily involved in biofilm maturation processes. It is conceivable that biofilm thickness is regulated by other genes such as SMU.881 (which was up-regulated), encoding GTFA sucrose phosphorylase.

This report of a global genomic expression profile describes modulation of the *S. mutans* transcriptome in the biofilm vs planktonic environment. The results presented here provide new insights at the molecular level into the gene expression of *S. mutans* when growing in biofilm conditions, which should lead to better understanding of the mechanism of biofilm formation by this species.

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Fig. 3. Live/dead staining of biofilms. *S. mutans* UA159 was grown at different biofilm depths in BHI, stained with LIVE/DEAD BacLight fluorescent dye and assessed by CLSM. The panels show the *xyz* and *xz* image reconstruction of cross-section images of 72 h (a) 100 μm and (b) 400 μm deep biofilms, constructed in the CDFF. Dead cells were stained red; live cells were stained green. The data presented are representative of three independent experiments.

Fig. 4. Regulation of gene expression during biofilm development examined by real-time RT-PCR. *S. mutans* UA159 was grown at biofilm depths of 100 μm (white bars) or 400 μm (black bars) in the CDFF. The data are expressed as the means $\pm$ SD of at least two biologically independent experiments. *Significant differences ($P < 0.05$) between gene expression in 100 μm vs 400 μm biofilms.

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