Differential response of *Streptococcus mutans* towards friend and foe in mixed-species cultures

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In the oral biofilm, the 'mitis' streptococci are among the first group of organisms to colonize the tooth surface. Their proliferation is thought to be an important factor required for antagonizing the growth of cariogenic species such as *Streptococcus mutans*. In this study, we used a three-species mixed culture to demonstrate that another ubiquitous early colonizing species, *Veillonella parvula*, can greatly affect the outcome of the competition between a pair of antagonists such as *S. mutans* and *Streptococcus gordonii*. Transcriptome analysis further revealed that *S. mutans* responds differentially to its friend (*V. parvula*) and foe (*S. gordonii*). In the mixed culture with *S. gordonii*, all but one of the *S. mutans* sugar uptake and metabolic genes were downregulated, while genes for alternative energy source utilization and H₂O₂ tolerance were upregulated, resulting in a slower but persistent growth. In contrast, when cultured with *V. parvula*, *S. mutans* grew equally well or better than in monoculture and exhibited relatively few changes within its transcriptome. When *V. parvula* was introduced into the mixed culture of *S. mutans* and *S. gordonii*, it rescued the growth inhibition of *S. mutans*. In this three-species environment, *S. mutans* increased the expression of genes required for the uptake and metabolism of minor sugars, while genes required for oxidative stress tolerance were downregulated. We conclude that the major factors that affect the competition between *S. mutans* and *S. gordonii* are carbohydrate utilization and H₂O₂ resistance. The presence of *V. parvula* in the tri-species culture mitigates these two major factors and allows *S. mutans* to proliferate, despite the presence of *S. gordonii*.

Received 17 January 2011

Revised 3 May 2011

Accepted 6 May 2011

INTRODUCTION

Biofilms in the human oral cavity are exceptionally diverse and can harbour more than 800 microbial species (Aas *et al.*, 2005; Keijser *et al.*, 2008; Paster *et al.*, 2001, 2006; Zaura *et al.*, 2009). Dental biofilm formation is hypothesized to be a sequential process (Diaz *et al.*, 2006; Kolenbrander *et al.*, 2006, 2010). After a new tooth emerges or a surface is cleaned, the surface is colonized by a group of bacteria named the 'pioneer colonizers', which are composed mostly of the 'mitis' streptococci (i.e. *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus mitis*, etc.). Subsequently, early colonizers such as

Streptococcus mutans and veillonellae, and bridging species such as the fusobacteria, join the community through interactions with the pioneer colonizers or by adhering to the available sites on the tooth surface. Growth of the early colonizers then modifies the local environment, making it favourable for the growth of late colonizers, which consist mostly of Gram-negative, obligate anaerobic bacteria. Eventually, through cell growth and co-adhesion, a mature biofilm is formed (for reviews, see Diaz *et al.*, 2006; Kolenbrander *et al.*, 2010). Once formed, the biofilm composition is relatively stable, featuring high biodiversity; however, environmental perturbation can disrupt this ecological balance, leading to the overgrowth of pathogens and the development of oral diseases (Marsh, 1994, 1999, 2006).

The 'mutans' streptococci (mainly *S. mutans* and *Streptococcus sobrinus*) are considered primary pathogens for the development of dental caries (tooth decay) (Loesche, 1986), while the mitis streptococci are benign, or even beneficial in preventing dental caries (Becker *et al.*, 2002; Caufield *et al.*, 2000). Both groups of bacteria reside in the same supragingival plaque, have nearly identical nutritional

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Abbreviations: CSP, competence-stimulating peptide; MLF, malolactic fermentation; TCA cycle, tricarboxylic acid cycle.

The microarray data discussed in this paper are available from Gene Expression Omnibus (GEO) under accession number GSE28259.

A supplementary table, listing differentially regulated genes in mixed cultures, is available with the online version of this paper.

requirements and are fierce competitors. Indeed, epidemiological studies have found an inverse relationship between the two groups of bacteria: high numbers of the mutans streptococci correlate with low numbers of the mitis streptococci, while colonization by the latter correlates with delayed colonization by the former (Becker *et al.*, 2002; Caufield *et al.*, 1993, 2000). Likewise, *in vivo* studies using germ-free rats have revealed a competitive exclusion between *S. mutans* and *S. sanguinis* (Mikx *et al.*, 1976). In our *in vitro* studies, we have shown that bacteriocins (mutacins) produced by *S. mutans* and H₂O₂ produced by *S. gordonii* or *S. sanguinis* are used as chemical weapons for interspecies competition. The winner of this competition depends upon which species occupies the niche first (Kreth *et al.*, 2005b).

Veillonellae are also early colonizers of the oral biofilm (Hughes *et al.*, 1988, 1992; Palmer *et al.*, 2006), and are one of the most prevalent colonizers of the human oral cavity (Dewhirst *et al.*, 2010). A common characteristic of the genus *Veillonella* is that they do not utilize carbohydrates; rather, they metabolize lactate, pyruvate and peptones to produce propionate and acetate (Rogosa & Bishop, 1964). This nutritional requirement makes them dependent upon the streptococci, which excrete lactate as a waste product of carbohydrate fermentation. *In vitro* and *in vivo* studies routinely demonstrate veillonellae coaggregating with streptococci (Hughes *et al.*, 1988, 1992; Palmer *et al.*, 2006). Of particular interest in this veillonellae–streptococci association is the removal of lactic acid by the veillonellae. Lactic acid produced by streptococci is primarily responsible for the characteristic demineralization found in dental caries, which raises an interesting question as to the role of veillonellae in cariogenesis. Early studies using gnotobiotic rats found reduced caries activity and tooth demineralization by *S. mutans* when co-inoculated with *Veillonella* species (Mikx *et al.*, 1972; Mikx & Van der Hoeven, 1975); however, more recent epidemiological studies in humans have found high numbers of veillonellae associated with high numbers of *S. mutans* in carious lesions (Aas *et al.*, 2008; Becker *et al.*, 2002; Rozkiewicz *et al.*, 2006; Russell *et al.*, 1990). *In vitro* studies also have found that co-culturing *S. mutans* with *Veillonella parvula* could increase the resistance of the former to antimicrobial substances (Luppens *et al.*, 2008). In this study, we further explore the molecular mechanism underlying these intricate interspecies relationships. We demonstrate that *V. parvula* alters the competitive outcome during interspecies competition between *S. mutans* and *S. gordonii*. Consistent with this result, we show that *S. mutans* exhibits a vastly different transcriptional responses in the presence of *V. parvula* and *S. gordonii*.

METHODS

Bacterial strains and growth conditions. *S. mutans* strain UA140, *S. gordonii* Challis and *V. parvula* PK1910 were used in this study. Strain UA140 was a clinical isolate from a severe caries lesion. *V.*

parvula strain PK1910 was formerly named *Veillonella atypica* PK1910 or *Veillonella* spp. PK1910 (Chalmers *et al.*, 2008; Hughes *et al.*, 1992), and is now renamed *V. parvula* PK1910 based upon our recent sequence analysis using the *rpoB* gene (Qi & Ferretti, 2011). Streptococcal species were routinely cultured in brain heart infusion (BHI) medium (Difco) or on BHI plates. *V. parvula* PK1910 was grown in BHI or Todd–Hewitt (TH) medium containing 0.6% sodium lactate (J. T. Baker). All cultures were grown either anaerobically (85% nitrogen, 5% carbon dioxide, 10% hydrogen) or aerobically (5% carbon dioxide) at 37 °C as static cultures.

Competition assay. Overnight, monocultures of all three species were centrifuged to remove the supernatants, and the cell pellets were resuspended in fresh TH broth. The cell suspension was adjusted to OD₆₀₀ 0.5, and mixed in specified ratios of *S. mutans*:*S. gordonii*, *S. mutans*:*V. parvula* and *S. mutans*:*S. gordonii*:*V. parvula*. The initial *S. mutans* concentration was set at ~10⁷ c.f.u. ml⁻¹ in all cultures. Mono-species, dual-species or triple-species cultures were then incubated aerobically as 10 ml static cultures in a 15 ml culture tube in the presence of 5% CO₂. Cell growth was monitored by plating. *S. mutans* was counted by plating on BHI plates with 1 mg spectinomycin ml⁻¹ (a spectinomycin-resistance gene was inserted in the chromosome of the UA140 strain), and *S. gordonii* was counted by plating on non-selective BHI plates, as its colonies are easily distinguished from those of *S. mutans*. *V. parvula* was counted by plating on BHI plates supplemented with 0.6% lactate and 12 µg tetracycline ml⁻¹. Growth curves were calculated based upon c.f.u. ml⁻¹, and the results were the mean of at least three experiments performed on different days.

Transcriptome analysis

Cell preparation. Mono- and mixed-species cultures were grown similarly to those in the competition assay. To harvest cells for the microarray, cell growth was monitored by measuring the OD₆₀₀ of the *S. mutans* monoculture. When the monoculture reached OD₆₀₀ 0.8 (late-exponential phase), cells were harvested by centrifugation and frozen at -80 °C until use.

RNA extraction and microarray. RNA isolation and microarray production were performed essentially as described previously (Wu *et al.*, 2010a), with the following addition. To ensure that equal amounts of *S. mutans*-specific RNA were used for all samples (mono- and mixed-species), real-time RT-PCR was used to quantify the *S. mutans* 16S rRNA in mixed-species samples using primers described previously (Wu *et al.*, 2010b) and the proportion of *S. mutans*-specific RNA was calculated.

Data analysis. Analysis of signal intensities was performed using the GeneChip operating system software (GCOS) version 1.4, and gene expression data were compared using the GCOS batch analysis function. Normalization procedures were performed directly by the software using a script designed by Affymetrix and provided with the *S. mutans* custom array. In addition to the general analysis required by the GeneChip, measures were also taken to address the specific issues associated with the mixed-culture microarray. For example, to assess the extent of cross-hybridization between *S. mutans* and *S. gordonii* and *V. parvula*, chromosomal DNA from the last two species was labelled and hybridized to the *S. mutans* GeneChip. Only rRNA, ribosome protein genes and some tRNA genes cross-hybridized with the *S. mutans* chip, especially for *S. gordonii*, and therefore these genes were eliminated from analysis. Additionally, after the initial data analysis with GCOS, the data were further normalized manually using the following steps: (1) the ratio of mixed: single species expression value for each gene was calculated with Microsoft Excel; (2) a mean ratio for each gene was calculated from four datasets; (3) a *t* test was used to calculate the *P* value of the fold-change for each gene using

the *gyrA* gene as reference. After this normalization, a cut-off of a twofold or greater change with a *P* value of ≤ 0.05 was used to generate a dataset of genes whose expression was altered in the mixed-species cultures as opposed to the single-species culture.

Real-time RT-PCR. Real-time RT-PCR was performed to validate the results generated by the microarray analysis. From the final list of twofold or greater responding genes, seven were randomly chosen to be further analysed. These seven genes represented a range of transcriptional responses as well as different functional groups. Primers were designed using Applied Biosystems Primer Express 3.0 software, which scans DNA sequences for primers suitable for comparative threshold value ($\Delta\Delta C_T$) analysis. Cells were cultured under the same conditions as described for the microarray. A 300 ng sample of total RNA was used for the analysis, essentially as described previously (Wu *et al.*, 2010a). The *gyrA* gene was used as the housekeeping gene reference, and all samples included a no-reverse-transcriptase control to assess genomic DNA contamination in the reactions.

RESULTS AND DISCUSSION

V. parvula increases the fitness of *S. mutans* in the mixed-species culture

As demonstrated in our previous studies (Kreth *et al.*, 2005b; Tong *et al.*, 2007), the mitis streptococci (i.e. *S. gordonii*, *S. sanguinis*, *Streptococcus Oligofermentans*, etc.) have the ability to severely inhibit *S. mutans* growth by producing large amounts of H_2O_2 . However, *S. mutans* is able to inhibit the mitis streptococci by producing bacteriocins. *In vitro*, the outcome of this inter-species competition depends on the environmental conditions and which species occupies the niche first (Kreth *et al.*, 2005b). Interestingly, *in vivo*, both *S. mutans* and the mitis streptococci can co-exist in the same biofilm community. We hypothesized that the presence of other species, such as veillonellae, might help to diffuse the conflict between the two competing groups of bacteria, resulting in their co-existence. To test this, we mixed monospecies cultures of *S. mutans*, *S. gordonii* and *V. parvula* at a ratio reminiscent of the early *in vivo* biofilm (~2% *S. mutans*, 10% *V. parvula* and 88% *S. gordonii*) (Mager *et al.*, 2003; Nyvad & Kilian, 1990; Rosan & Lamont, 2000). The mixed cultures were incubated as static cultures under aerobic conditions in the presence of 5% CO_2 , a condition that mimics the oral cavity environment. Samples were taken every 2 h for viable cell counts. As shown in Fig. 1, in the *S. mutans*–*S. gordonii* dual-species culture, the growth of *S. mutans* was severely inhibited, as the growth rate and final biomass were reduced by 35 and 85%, respectively. Interestingly, when *V. parvula* was introduced into the dual-species culture, the growth of *S. mutans* was rescued, resulting in a growth rate and final biomass similar to those of the *S. mutans* monoculture. This result suggested that *V. parvula* could alter the outcome of the competition between *S. mutans* and *S. gordonii*. It is important to note that in binary cultures with *S. mutans*, both *S. gordonii* and *V. parvula* grew as well as in their respective monocultures, while in tri-species cultures, *S. gordonii* achieved higher

Fig. 1. Growth of *S. mutans* (Sm) in dual-species culture with *S. gordonii* (Sg) and in tri-species cultures with *V. parvula* (Vp). The experimental setup is described in Methods. Each experiment was repeated at least three times in triplicate. Presented is a typical result. *K* is the growth rate, calculated using the formula $K = (\ln N - \ln N_0) / (t - t_0)$, where *N* is the c.f.u. ml⁻¹ at time *t*, and *N*₀ is the c.f.u. ml⁻¹ at time *t*₀.

yields than in mono-species and dual-species cultures with *S. mutans* (data not shown).

S. mutans responds differentially to *V. parvula* and *S. gordonii*

To further explore the mechanism of this interesting interspecies interaction, microarray studies were performed using *S. mutans* monocultures (Sm), *S. mutans*–*S. gordonii* dual cultures (Sm + Sg), *S. mutans*–*V. parvula* dual cultures (Sm + Vp) and *S. mutans*–*S. gordonii*–*V. parvula* tri-cultures (Sm + Sg + Vp). The *S. mutans* response to co-culturing with *S. gordonii* and *V. parvula* was dramatically different. When a cut-off of at least twofold with a *P* value of ≤ 0.05 was taken, a total of 400 genes were affected by coculturing with *S. gordonii* (Supplementary Table S1 and Table 2). In contrast, only 54 genes were differentially expressed by growth with *V. parvula*. In the *S. mutans*–*S. gordonii*–*V. parvula* tri-species culture, the total number of affected genes (354 total) was more similar to that of the *S. mutans*–*S. gordonii* culture. No gene was shared between the Sm + Sg and the Sm + Vp mixed cultures, indicating a completely different response of *S. mutans* toward the other two species (Fig. 2). In contrast, among the 54 genes affected in the Sm + Vp cultures, 15 were shared with the tri-species culture, suggesting that *V. parvula* could still influence *S. mutans* gene expression in a similar manner, even when *S. gordonii* was present. Additionally, 22 genes were similarly affected in all mixed cultures, indicating a non-specific response (Fig. 2). Interestingly, more genes (63.5%) were downregulated in the Sm + Sg culture than in the Sm + Vp culture (51.8%), and about one-third of the downregulated genes in the Sm + Sg culture were those involved in energy metabolism and biosynthesis. In

Fig. 2. Venn diagram of *S. mutans* genes affected in the three mixed-species cultures. Numbers were calculated based on genes listed in Supplementary Table S1. Only genes that were affected similarly (in the same trend) were treated as shared; those that were affected in opposite directions were treated as unique or not shared. Abbreviations: Sm, *S. mutans*; Sg, *S. gordonii*; Vp, *V. parvula*.

contrast, <20% of the downregulated genes in the Sm + Vp mixed culture belonged to this functional class.

To confirm the microarray results, real-time RT-PCR was used to measure the expression of seven genes, SMU.152, SMU.308, SMU.423, SMU.910, SMU.932, SMU.961 and SMU.1946, randomly chosen from different functional groups with different levels of response. Most of these genes exhibited the same trend of response as in the microarray (Table 3), especially for the Sm + Sg and the tri-species cultures, suggesting that the microarray results were likely to be an accurate reflection of the gene responses of *S. mutans* to growth in mixed cultures. Taken together, these results suggested an overall reprogramming of gene expression in *S. mutans* when co-cultured with *S. gordonii*, consistent with the observed growth inhibition of *S. mutans* by *S. gordonii* (Fig. 1). In contrast, *V. parvula* had little effect on either the growth or the gene expression of *S. mutans* in dual-species cultures; however, in tri-species culture, it had a significant effect on the gene expression pattern of *S. mutans*, which probably accounted for the observed growth rescue of the latter.

The most downregulated genes in co-cultures with *S. gordonii*

The most conspicuous downregulated genes encoded sugar transporters and their related metabolic enzymes. In fact, all sugar transporters, except one (SMU.2047c, EII^{glu}), known to be present in the *S. mutans* genome (Ajdić *et al.*, 2002; Ajdić & Pham, 2007), were downregulated.

SMU.1957c–1961c encode fructose/mannose EII subunits A–D (Ajdić & Pham, 2007), and are located in the same operon as SMU.1956c, a membrane protein of unknown function. In our microarray, all genes in the operon, except SMU.1958c, were downregulated two- to threefold (SMU.1958c was downregulated 1.6-fold). SMU.1841c encodes the sucrose phosphotransferase subunits EIIABC, while SMU.1843 and 1844, encode a sucrose-6-phosphate hydrolase and sucrose operon regulator, respectively. All three genes were downregulated three- to fivefold. SMU.1490c–1498c encode genes for lactose transport and metabolism. Although SMU.1490c (phospho- β -D-galactosidase) did not meet the cut-off (twofold change with a *P* value of <0.05), all other genes in the operon were downregulated 2.5- to threefold. In addition, genes for the transport or metabolism of minor sugars and sugar alcohols were also downregulated, such as those for ribose (SMU.2142c), maltose (SMU.1568–1571), cellobiose (SMU.1596c–1600c), ribulose (SMU.270–272), sorbitol (SMU.308–314) and mannitol (SMU.1182c–1185c). Taken together, these results demonstrated that in the mixed culture with *S. gordonii*, *S. mutans* energy metabolism was suppressed. Consequently, genes involved in macromolecule biosynthesis and cell division were also downregulated (Table 1), resulting in a slower growth rate and a lower cell mass, as shown in Fig. 1.

One possibility is that the downregulation of sugar transporters and energy metabolism genes by *S. gordonii* is due to carbohydrate limitation in the mixed culture, as *S. gordonii* was numerically dominant over *S. mutans* (44:1). It is conceivable that this numerical advantage may have simply given them an advantage in consuming the easily metabolizable carbohydrates. The data from the tri-species culture suggested that this might be the case for the three major sugars, fructose, lactose and sucrose, while a different mechanism might explain the effect on the transport and utilization of the minor sugars and sugar alcohols (see discussion below of the genes uniquely affected in the tri-species cultures). For example, the downregulation of sugar transporters and energy metabolism genes could be due to feedback inhibition as a result of reduced glycolytic rates, similar to the feedback inhibition of growth by carbohydrate starvation observed in *Lactococcus lactis* (Ganesan *et al.*, 2007). Since *S. gordonii* produces H₂O₂, this may inhibit *S. mutans* growth by poisoning the glycolysis system [i.e. inhibition of glyceraldehyde-3-phosphate dehydrogenase (Baldeck & Marquis, 2008)]. This would result in accumulation of intermediates and a reduction in ATP synthesis due to the diminished downstream activities of the glycolysis pathway. Presumably, the accumulation of intermediates could also affect the expression of various metabolic genes through feedback inhibition. With a decrease in energy production, we would expect a concomitant decrease in the growth rate and final yield of *S. mutans*, as shown in Fig. 1. Accordingly, genes related to biosynthesis and cell division were downregulated as well (Table 1). Despite the

Table 1. Differentially regulated genes discussed in the text

Abbreviations: mg, *S. mutans* + *S. gordonii* mixed culture; m, *S. mutans* single-species culture; mv, *S. mutans* + *V. parvula* mixed culture; mvg, *S. mutans* + *V. parvula* + *S. gordonii* mixed culture.

Gene (SMU.)	mg/m	mv/m	mvg/m	Description	Functional class
55			251.0	Hypothetical protein	Unknown
56			8.71	Conserved hypothetical protein	Unknown
137	0.40	0.08	0.26	Malolactic enzyme	Energy metabolism
138		0.08	0.39	Putative malate permease	Transport
139		0.13		Oxalate decarboxylase	Unknown
140		0.12		Glutathione reductase	Stress response
141		0.09	0.45	Conserved hypothetical protein, membrane protein	Unknown
150	0.16		0.19	Bacteriocin mutacin IV (<i>nlmA</i>)	Stress response
151	0.17		0.24	Bacteriocin mutacin IV (<i>nlmB</i>)	Stress response
152	0.08		0.02	Hypothetical protein, membrane protein	Unknown
153	0.13		0.21	Hypothetical protein, small peptide	Unknown
270	0.41*	0.41	0.42	Ribulose-monophosphate PTS pathway enzyme IIC	Sugar transport
271	0.48			Putative PTS system, enzyme IIB component	Sugar transport
308	0.20		0.40	Sorbitol-6-phosphate 2-dehydrogenase	Energy metabolism
423	0.05	0.23	0.05	Bacteriocin mutacin VI, <i>nlmD</i>	Stress response
437c			4.75	Conserved hypothetical protein	Unknown
663	2.02		2.79	<i>N</i> -Acetyl- γ -glutamyl-phosphate reductase (<i>N</i> -acetyl-glutamate- γ -semialdehyde dehydrogenase)	Amino acid biosynthesis, glutamate family
664	5.12		7.06	Ornithine acetyltransferase/ <i>N</i> -acetylglutamate synthase	Amino acid biosynthesis, glutamate family
665	4.69		5.19	Acetylglutamate kinase	Amino acid biosynthesis, glutamate family
666	4.33		4.72	<i>N</i> -Acetylornithine aminotransferase	Amino acid biosynthesis, glutamate family
730		4.63		<i>abrB</i> -like transcription regulator	Regulation
731		3.79		ABC transporter, ATP-binding protein	Transport
732	0.49	3.97		Membrane protein	Unknown
870	0.41		0.40	Lactose phosphotransferase system repressor/transcriptional repressor of the fructose operon, <i>fruR</i>	Regulation
872	0.52	0.44	0.45	Putative PTS system, fructose-specific enzyme IIABC component, <i>fruA</i>	Fructose transport
877	0.39			α -Galactosidase	Energy metabolism
878	0.44*			ABC transporter, sugar-binding protein, MsmE	Sugar transport
879	0.38			Sugar-binding ABC transporter, permease protein MsmF	Sugar transport
880	0.40			Sugar-binding ABC transporter, permease protein MsmG	Sugar transport
932	17.36			Hypothetical protein	Unknown
933	19.14			Amino acid ABC transporter, substrate-binding	Transport
934	21.7			Amino acid ABC transporter, permease protein	Transport
935	25.3			Amino acid ABC transporter, permease protein	Transport
936	9.38			Amino acid ABC transporter, ATP-binding protein	Transport
981			3.27	β -Glucosidase, BglB	Energy metabolism
982			3.42	β -Glucosidase, BglB protein	Energy metabolism
1010c	4.80		9.04	Citrate lyase synthetase, <i>citC</i>	Energy metabolism
1011c	3.23		6.98	CitG protein	Energy metabolism
1012c	2.77		5.06	Transcriptional regulator	Regulation
1013c	4.00		5.60	Mg ²⁺ /citrate complex transporter	Transport
1148	3.68		9.15	ABC transporter, ATPase	Transport
1149	4.05		7.48	ABC transporter, transmembrane domain	Transport
1150	4.41		8.40	ABC transporter, transmembrane domain	Transport
1183c	0.48*			PTS system, COG1762, mannitol/fructose-specific IIA	Sugar transport
1184c	0.52*			Transcriptional regulator, <i>mtlR</i>	Regulation
1185c	0.29			PTS system, COG2213, mannitol-specific IIBC component	Sugar transport
1410	4.00		6.15	Fumarate reductase	Energy metabolism
1411	4.03		5.59	Conserved hypothetical protein; COG0477, membrane protein	Transport

Table 1. cont.

Gene (SMU.)	mg/m	mv/m	mvg/m	Description	Functional class
1492c	0.39		0.53*	PTS system, cellobiose-specific IIA component	Sugar transport
1493c	0.43			Tagatose-1,6-bisphosphate aldolase	Energy metabolism
1495c	0.31		0.39	Galactose-6-phosphate isomerase, LacB	Energy metabolism
1496c	0.32			Galactose-6-phosphate isomerase, LacA	Energy metabolism
1498c	0.34		0.47	Lactose phosphotransferase system repressor, LacR	Energy metabolism
1519	3.86		3.45	Putative amino acid ABC transporter, ATP-binding protein	Transport
1520	3.55		3.13*	Putative ABC transporter, glutamate-binding protein	Transport
1521	2.86		2.17	Glutamate ABC transporter, permease	Glutamate transport
1522	2.15		1.73	Glutamate ABC transporter, permease	Glutamate transport
1568	0.10		0.32	Maltose maltodextrin-binding protein MalE	Sugar transport
1569	0.19		0.39	Maltodextrin ABC transporter, permease protein MalF	Sugar transport
1570	0.22		0.33	Maltose maltodextrin ABC transporter, MalG permease	Sugar transport
1571	0.17		0.30	Maltose ABC transporter, ATP-binding protein, MsmK	Sugar transport
1574c			4.03	Conserved hypothetical protein	Unknown
1597c	0.31			Conserved hypothetical protein, membrane protein	Unknown
1598c	0.35*			Putative PTS system, cellobiose-specific IIA component, CelC	Cellobiose uptake
1599c	0.35*			Transcriptional regulator, CelR	Regulation
1600c	0.12		0.27	PTS system, cellobiose-specific IIB component, CelB	Cellobiose uptake
1818c			7.86	Hypothetical protein	Unknown
1841c	0.30		0.46	PTS system, sucrose-specific IIBC component	Sugar transport
1843	0.16		0.23	Sucrose-6-phosphate hydrolase	Energy metabolism
1844	0.26		0.38	Sucrose operon repressor	Regulation
1897	4.42		2.50	ABC transporter, ATP-binding protein; similar to BlpA	Transport
1898	3.05		3.91	ABC transporter, ATP-binding and permease element	Transport
1899	4.17		4.90	ABC transporter, ATP-binding and permease protein	Transport
1900	4.32		5.81	ABC transporter	Transport
1902c	0.40		0.40	Bacteriocin-like peptide	Unknown
1903c	0.18	0.34	0.20	Bacteriocin-like peptide	Unknown
1904c	0.25	0.53*	0.32	Possible bacteriocin immunity protein	Unknown
1905c	0.21	0.49	0.29	CSP-regulated bacteriocin-like peptide	Unknown
1906c	0.12	0.25	0.08	CSP-regulated bacteriocin-like peptide	Unknown
1956c	0.32		0.41	Hypothetical protein, membrane protein	Unknown
1957c	0.47		0.58	Fructose-specific enzyme IID component	Sugar transport
1960c	0.40		0.61	Fructose-specific enzyme IIB component	Sugar transport
1961c	0.29		0.49	Fructose-specific IIA component	Sugar transport
2142c	2.00		2.15	COG0698, ribose-5-phosphate isomerase	Energy metabolism

*Denotes genes with a P value ≥ 0.05 but ≤ 0.1 . These genes were included in the table only when other members of the same operon exhibited similar changes in gene expression and met the P value < 0.05 cut-off.

ubiquitous effect upon sugar transport operons, it was surprising to observe that the putative glucose transporter (SMU.2047c) was not affected. SMU.2047c has been annotated as a glucose transporter based on its homology with the EII^{glc} of *Bacillus subtilis* (Abranches *et al.*, 2006); however, inactivation of this gene did not affect cell growth in medium containing glucose as the carbon source (Abranches *et al.*, 2006). Our results appear to be consistent with this observation.

The most important upregulated genes in co-cultures with *S. gordonii*

While *S. mutans* growth rate was reduced in the mixed culture with *S. gordonii*, its growth persisted (Fig. 1), which suggests that *S. mutans* likely used alternative energy

sources to compensate for its reduced capacity to metabolize carbohydrates. Inspection of the upregulated genes in the dataset (Table 1) may provide further evidence of this. We identified four upregulated operons involved in generating energy from alternative sources: the citrate lyase synthetase operon (SMU.1010c–1013c), the fumarate reductase operon (SMU.1410–1411), the glutamate transporter operon (SMU.1519–1522) (Krastel *et al.*, 2010) and the glutamate family amino acid synthesis operon (SMU.663–666). All genes in these operons were upregulated three- to fivefold in the mixed culture with *S. gordonii*, although SMU.1520 had a slightly higher P value (0.06). SMU.1010c encodes citrate lyase synthetase. Citrate lyase catalyses the cleavage of citrate to acetate and oxaloacetate; the latter is an intermediate of the tricarboxylic acid (TCA) cycle, and can be further metabolized

Table 2. Summary of differentially regulated genes in different functional classes

Abbreviations: Sm, *S. mutans*; Sg, *S. gordonii*; Vp, *V. parvula*. Down, downregulation; up, upregulation.

Functional class	Sm + Sg		Sm + Vp		Sm + Sg + Vp	
	Down	Up	Down	Up	Down	Up
Energy metabolism	32	8	4	1	23	10
Biosynthesis/central metabolism	45	27	1	4	37	18
Cell division	10	3	0	2	5	5
Sugar transport	16	0	2	0	13	0
Transport	9	32	4	3	11	30
Regulation	31	5	1	4	25	12
Stress response	14	7	2	2	14	8
Adhesion	1	4	1	2	0	4
Unassigned	10	6	0	2	6	9
Unknown	84	56	12	7	69	69
Total	252	148	28	26	203	161

to enter into the pyruvate metabolic pathway of the glycolysis cycle or for amino acid synthesis (Korithoski *et al.*, 2005) (Fig. 3). Citrate lyase is post-translationally activated, and its activation requires citrate lyase synthetase and the CitG protein (encoded by SMU.1011c) (Schneider *et al.*, 2000). SMU.1012c encodes a transcription regulator, which is probably responsible for the regulation of the operon, and SMU.1013c encodes a Mg^{2+} /citrate complex transporter. Thus, by increasing citrate transport and activating citrate lyase activity, *S. mutans* could use citrate as an alternative energy source. In lactic acid bacteria, citrate has been used to generate proton motive force and to increase acid tolerance (Garcia-Quintans *et al.*, 1998; Ramos *et al.*, 1994, 1995). In *S. mutans*, citrate has been shown to be transported and metabolized, though not as a principal carbon source (Korithoski *et al.*, 2005). Moreover, preincubation with citrate increases *S. mutans*

survival at acidic pH (Korithoski *et al.*, 2005). Our results suggested that citrate transport and metabolism may play an important role in *S. mutans* persistence in the early biofilm, where the mitis streptococci are numerically dominant (Rosan & Lamont, 2000).

The possible function of glutamate transport (SMU.1519–1522) and glutamate family biosynthesis genes in *S. mutans* survival is illustrated in Fig. 3. After glutamate is transported into the cell by the gene products of SMU.1519–1522, it could be acetylated by SMU.664 to *N*-acetyl glutamate, which is then phosphorylated by SMU.665 to form *N*-acetyl glutamate phosphate. *N*-acetyl glutamate phosphate is then reduced by the gene product of SMU.663 to *N*-acetyl glutamate phosphate semialdehyde, which is converted to *N*-acetyl ornithine by the gene product of SMU.666. Acetyl ornithine can enter the urea cycle, through which it is converted into arginine and fumarate. Arginine can be used directly for protein synthesis, while fumarate can be converted to succinate by fumarate reductase, the gene product of SMU.1610. Succinate is an intermediate of the TCA cycle, through which intermediate metabolites and ATP can be generated. Although *S. mutans* does not have a complete TCA cycle, a partial TCA cycle is present (Ajdić *et al.*, 2002). Interestingly, glutamate has also been found to play an important role in regulating the activity of citrate lyase in *Clostridium sphenoides* (Antranikian & Gottschalk, 1989). Although the effect of glutamate on citrate lyase activity in *S. mutans* is not known, a recent study found that the glutamate transport operon plays an important role in acid tolerance of *S. mutans* (Krstel *et al.*, 2010), suggesting a possible link with citrate metabolism (Korithoski *et al.*, 2005). Thus, these four upregulated operons may actually form a network linking the transport and metabolism of glutamate and citrate with three major metabolic cycles (the urea cycle, the TCA cycle and glycolysis) to generate energy and metabolic intermediates for *S. mutans* when sugar metabolism is suppressed (Fig. 3).

Table 3. Comparison of microarray and real-time RT-PCR results for selected genes

Abbreviations: Sm, *S. mutans*; Sg, *S. gordonii*; Vp, *V. parvula*.

Gene	Sm + Sg/Sm (fold change)		Sm + Vp/Sm (fold change)		Sm + Sg + Vp/Sm (fold change)	
	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
SMU.152	−12.5*	−6.0	NC†	−1.2	−50.0	−4.0
SMU.308	−5.0	−11.9	NC	−26.5	−2.5	−46.7
SMU.423	−20.0	−3.6	−4.2	−1.0	−20.0	−4.7
SMU.910	+4.1‡	+14.0	+2.5	+1.6	+3.6	+1.1
SMU.932	+17.4	+16.5	NC	+1.6	NC	−1.3
SMU.962	+6.7	+12.7	+2.5	+1.4	+3.8	+1.1
SMU.1946	+3.2	+2.3	NC	+2.7	+3.0	+2.0

*Minus value: downregulated in mixed culture.

†NC, No change.

‡Plus value: upregulated in mixed culture.

Fig. 3. Proposed pathways upregulated in the *S. mutans* + *S. gordonii* mixed cultures. Genes in bold type are upregulated in the mixed culture; genes in plain type exist in the *S. mutans* genome, but were not upregulated in the microarray. The pathways are proposed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<http://www.genome.jp/kegg/pathway.html#energy>).

Another set of significantly upregulated genes was the cysteine transport operon (SMU.932–936) (Sperandio *et al.*, 2010), which was upregulated 10–25 fold (Table 1). Cysteine is an essential nutrient for *S. mutans* growth (Terleckyj & Shockman, 1975). Cysteine can be converted to methionine by cystathionine γ -synthase (SMU.1675) and homocysteine *S*-methyltransferase (SMU.873 and 874). It can also be used in a thiol form for *S. mutans* H₂O₂ resistance (Thomas *et al.*, 1983). In our case, we propose that cysteine is more likely to be used for the latter purpose, as H₂O₂ production by *S. gordonii* (or *S. sanguinis*) has been shown to be the primary inhibitor of *S. mutans* growth in mixed cultures (Kreth *et al.*, 2005b). In addition to the cysteine transporter, two more operons encoding ABC transporters were also upregulated (SMU.1148–1150, SMU.1897–1900). Interestingly, SMU.1897–1900 have been shown to encode the transporter for the secretion of CSP (Hale *et al.*, 2005), while SMU.1148–1150 exhibit homology to genes of lantibiotic peptide ABC transporters. In our previous study, we have shown that CSP controls the expression of a group of bacteriocin genes whose products specifically kill the mitis streptococci, such as *S. gordonii*, to

cause DNA release for uptake by competent *S. mutans* cells (Kreth *et al.*, 2005a). Wang & Kuramitsu (2005) demonstrated that to fight back, *S. gordonii* produces a protease that degrades CSP, thus downregulating bacteriocin production genes in *S. mutans*. Our results here suggest that *S. mutans* may further counter this strategy by increasing CSP export.

The most affected genes in co-cultures with *V. parvula*

As shown in Table 1, few genes were affected in the Sm + Vp mixed culture more than threefold. One operon that stands out as the most downregulated encodes genes for malate transport and metabolism (SMU.137–141). All genes in this operon were downregulated ~10-fold. Malolactic fermentation (MLF) is a form of secondary metabolism carried out by lactic acid bacteria, including *S. mutans*, to produce ATP. The dicarboxylic acid L-malate is converted to the monocarboxylic acid lactate and CO₂ by the malolactic enzyme (encoded by SMU.137). Since malic acid is a stronger acid than lactic acid, this reaction creates

a pH gradient between the cytoplasm and the outside environment, which drives ATP synthesis by the $F(H^+)ATPase$ (Salema *et al.*, 1996; Sheng & Marquis, 2007). In *S. mutans* it has been shown that MLF is induced by low pH and the presence of L-malate (Sheng & Marquis, 2007), and accordingly the gene expression of SMU.137–140 is also induced by low pH and the presence of malate in the growth medium (Lemme *et al.*, 2010). In batch culture, the expression of this operon appears to follow the growth curve, with expression levels peaking at early stationary phase (Lemme *et al.*, 2010), when the growth medium had been acidified. Since our samples for microarray analysis were taken at late-exponential phase, we suspect that the malolactic operon in the monoculture was induced by the low pH at this stage. Thus, the downregulation of the malolactic operon in the Sm + Vp mixed-species culture could be due to the higher pH in the mixed culture because of the consumption of lactic acid by *V. parvula*. Indeed, the pH of the *S. mutans* monoculture at late-exponential phase was found to be approximately 5.4, while in the mixed culture it was approximately 6.4 (unpublished data). Thus, the higher pH of the Sm + Vp culture presumably resulted in lower expression of the malolactic enzyme operon.

The most significantly upregulated genes in the Sm + Vp mixed culture were SMU.730–732, which constitute a single operon and are upregulated approximately fourfold. SMU.730 encodes a 70 aa protein with an AbrB-like transcription regulator domain. SMU.731 and 732 encode an ABC transporter of unknown function. At present, the function of this operon and its relationship to mixed-species growth are unknown.

Genes uniquely affected in the tri-species culture

Despite the large overlap between the Sm + Sg and tri-species microarray datasets (Fig. 2), we identified some important differences between them that may provide some insight into the improved growth of *S. mutans* in the tri-species culture. The most conspicuous change in gene expression pattern in the tri-species culture was the upregulation of five operons involved in sugar transport and metabolism: the raffinose transport system (SMU.877–880), the mannitol transport operon (SMU.1183c–1185c), the maltose/maltodextrin transporters (SMU.1568–1571), the cellobiose uptake system (SMU.1597c–1599c) and the β -galactosidase genes (SMU.981–982). For the raffinose transport system, the mannitol transport operon and the cellobiose uptake system, the gene expression level in the tri-species culture was the same as in the Sm monospecies culture, while in the Sm + Sg dual-species culture, these genes were all downregulated two- to threefold (Table 1). For the maltose/maltodextrin transporters and the β -galactosidase genes, expression increased 1.6- to threefold in the tri-species culture with respect to the dual-species culture. Interestingly, genes for the transport and utilization of major sugars, such as sucrose (SMU.1841–1844), fructose/mannose (SMU.1957–1961)

and lactose (SMU.1490–1498), were not upregulated in the tri-species culture. This suggested that the presence of *V. parvula* in the mixed-species culture somehow increased the preference of *S. mutans* for the minor sugars and sugar alcohols. In addition, the citrate operon (SMU.1010c–1013c), which was already upregulated in the Sm + Sg dual-species culture, was increased even further by an additional 1.5- to twofold in the tri-species culture, further boosting the capacity of *S. mutans* for utilizing alternative energy sources.

In addition to the increased expression of various sugar transporters and the citrate operon, the tri-species culture also specifically increased the expression of several hypothetical genes. For example, SMU.55 and SMU.56 were upregulated >250-fold and over eightfold, respectively, in the tri-species culture. In fact, these genes exhibited little or no detectable signal in both the mono- and dual-species cultures, suggesting they were uniquely expressed in the tri-species condition. SMU.55 encodes an 87 aa protein of unknown function, and SMU.56 encodes a streptococcus-specific peptide of 42 aa; both genes appear to be co-transcribed. Additionally, several genes encoding hypothetical proteins (SMU.437c, SMU.955, SMU.1574c and SMU.1818c) were uniquely upregulated more than fourfold in the tri-species culture (Table 1). Whether these genes play any role in the interspecies interaction awaits further experimentation.

Genes commonly affected in all mixed-species cultures

Among the 22 commonly affected genes in all mixed cultures, a group of bacteriocin genes are worth noting. SMU.423 (*nldD*), 1905c and 1906c encode putative bacteriocins (Xie *et al.*, 2010) and 1904c encodes a putative bacteriocin immunity/modification protein, all of which have been shown to be regulated by CSP in *S. mutans* (Kreth *et al.*, 2006; van der Ploeg, 2005; Perry *et al.*, 2009). *S. gordonii* has been shown to downregulate CSP-induced bacteriocin genes of *S. mutans* by degrading CSP with a peptidase (Wang & Kuramitsu, 2005). This suggests that the downregulated bacteriocin-like genes could be a result of CSP degradation by *S. gordonii* in the mixed culture. Bacteriocin production is also a stress response of *S. mutans* (Kreth *et al.*, 2005a, b; Qi *et al.*, 2001), which suggests that the downregulation of bacteriocin gene expression in the mixed culture with *V. parvula* is an indication of no stress for *S. mutans*. In fact, *S. mutans* grows equally well in the spent medium of *V. parvula* as in fresh medium, and slightly better in mixed culture with *V. parvula* than in monoculture (data not shown), further supporting the notion that a mutualistic relationship exists between *S. mutans* and *V. parvula*.

Ecological implications of this study

How different species co-exist in a multispecies ecosystem is a particularly important aspect of polymicrobial disease

Fig. 4. Summary of *S. mutans* responses in mixed cultures. Blocked lines, downregulated; arrowed lines, upregulated. See text for details.

aetiology (Bakaletz, 2004; Brogden *et al.*, 2005; Rogers *et al.*, 2010; Sibley *et al.*, 2006). In this study, we used three-species mixed cultures as a facile model of the early microbial community of the supragingival plaque. Our results demonstrate that introducing a 'friendly' species into a mixed culture of competitors could affect this competition, resulting in the co-existence of all three species. Using a microarray analysis we further demonstrate that *S. mutans* responds differentially to its friend (*V. parvula*) and foe (*S. gordonii*). As illustrated in Fig. 4, when engaged in competition with *S. gordonii*, *S. mutans* sugar uptake and metabolic genes are downregulated, resulting in slower growth and a lower cell mass. To remain persistent in the mixed culture, *S. mutans* upregulated genes for alternative energy source acquisition and utilization, as well as genes to deal with environmental stress (H_2O_2) in the mixed culture. Thus, in the mixed culture with *S. gordonii*, nutrition and stress appear to be the major factors affecting *S. mutans* fitness. In co-cultures with *V. parvula*, the consumption of lactic acid appears to abolish the requirement for MLF, since *S. mutans* strongly downregulated these genes. Presumably, this is due to the increased pH of the culture. When *V. parvula* was introduced into the mixed culture of the two competitors, *S. mutans* growth inhibition was rescued, and genes for the uptake and metabolism of minor sugars were upregulated, while genes required for H_2O_2 stress (cysteine transporters) were downregulated. Thus, *V. parvula* appeared to promote *S. mutans* growth in the tri-species culture by mitigating the two major factors that limit *S. mutans* growth in the presence of *S. gordonii*. Although the exact mechanism by which *V. parvula* promoted *S. mutans* growth needs to be further explored, our results at least provide a reasonable explanation for the numerous *in vivo* observations that veillonellae appear to be associated with carious lesions despite their consumption of lactic acid produced by cariogenic bacteria.

ACKNOWLEDGEMENTS

We thank Dr Zhiyuan Chen for helping with the microarray data deposition. This work was supported in part by NIH grants R15 DE019940 to F.Q. and a Centers of Biomedical Research Excellence (COBRE) P20-RR018741-05 grant to J.M.

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Edited by: M. Whiteley