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Transcriptional analysis of *gtfB*, *gtfC*, and *gbpB* and their putative response regulators in several isolates of *Streptococcus mutans*

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Background: *Streptococcus mutans*, a major dental caries pathogen, expresses several virulence genes that mediate its growth, accumulation on tooth surfaces, and acid-mediated tooth demineralization. GtfB and GtfC catalyze the extracellular synthesis of water-insoluble glucan matrix from sucrose, and are essential for accumulation of bacteria in the dental biofilm. GbpB, an essential protein of *S. mutans*, might also mediate cell-surface interaction with glucan.
Aim/methods: In this study, we determined the transcription levels of *gtfB*, *gtfC*, and *gbpB*, and several putative transcriptional response regulators (*rr*) at different phases of planktonic growth in 11 *S. mutans* strains.
Results: Activities of *gtfB* and *gtfC* were growth-phase dependent and assumed divergent patterns in several strains during specific phases of growth, while *gbpB* activities appeared to be under modest influence of the growth phase. Transcription patterns of the *rr vicR*, *covR*, *comE*, *ciaR*, and *rr1* were growth-phase dependent and some of these genes were expressed in a highly coordinated way. Each *rr*, except *comE*, was expressed by all the strains. Patterns of virulence and regulatory genes were, however, strain-specific.
Conclusions: The findings suggest that mechanisms controlling virulence gene expression are variable among genotypes, providing the notion that the genetic diversity of *S. mutans* may have important implications for understanding mechanisms that regulate the expression of virulence genes in this species.

Key words: gene expression; genetic diversity; glucan-binding protein; glucosyltransferase; *Streptococcus mutans*; two-component system

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Streptococcus mutans is one of the main species involved in dental caries in humans. Its virulence is, in part, a result of the expression of virulence factors, which allow these cariogenic microorganisms to accumulate in the dental biofilm under highly acidic conditions that lead to tooth demineralization. The most widely studied virulence trait of *S. mutans* is its accumulation in the dental biofilm mediated by extracellular glucan synthesized from sucrose by glucosyltransferase (Gtf). Three distinct Gtfs (GtfB, GtfC, and GtfD) produced by *S. mutans* catalyze the synthesis of extracellular glucan. These Gtfs are encoded, respectively, by *gtfB*, *gtfC*, and *gtfD* (2). GtfB and GtfC are important for virulence (22) because of the high degree of insolubility of their glucan products, while GtfD synthesizes water-soluble glucan [reviewed in ref. (2)]. Interaction of the *S. mutans* cell surface with the extracellular matrix of insoluble glucan is not well understood, but it is suggested that this process involves glucan-binding proteins (Gbps). Four distinct Gbps have been identified in *S. mutans*. These Gbps, GbpA, GbpB, GbpC, and GbpD, are encoded by *gbpA*, *gbpB*, *gbpC*, and *gbpD*, respectively. The Gbps comprise a heterogeneous group of proteins that can be secreted and/or cell-

associated (GbpA, GbpB and GbpD) or covalently linked to the cell wall (GbpC) [reviewed in ref. (2)]. The specific biological role of each Gbp is still uncertain, and their association with virulence remains to be investigated further (2, 16). GbpB appears to be associated with *S. mutans* virulence because immunization with this protein confers protection against dental caries in animal models (29).

The mechanisms that regulate the expression of *gtfB*, *gtfC*, and *gbpB* are still unknown but it appears that these genes are under the control of two-component transduction systems (3, 27). These two-component transduction systems are formed by a membrane histidine kinase that undergoes autophosphorylation once it recognizes an environmental signal. The phosphate group is then transferred to its cognate response regulator (RR), which in turn binds DNA at specific sites to regulate the expression of genes under its control. The genome of *S. mutans* strain UA159 includes 13 two-component transduction systems (1). Five of these were recently associated with biofilm accumulation and/or structure, and/or expression of *gtfB*, *gtfC*, and *gbpB*. These include *comD/comE*, *rr11*/*rh11*, *ciaH/ciaR*, *vicK/vicR*, and *covR*, the last of which encodes an orphan RR (1, 3, 11, 14, 15, 25, 27). These systems appear to comprise a complex signaling network, and it is not known whether some of these RR are directly or indirectly activated by the same environmental signal. Another important issue in understanding the regulation of virulence gene expression in *S. mutans* is potential strain variability (19, 21). To investigate variable expression of virulence genes, we determined the transcription levels of the genes responsible for glucan synthesis, *gtfB*, *gtfC*, *gbpB*, and the regulatory genes *vicR*, *covR*, *comE*, *rr11*, and *ciaR*, at different phases of planktonic growth in several *S. mutans* clinical genotypes and laboratory strains.

Material and methods

Strains and culture conditions

Eleven *S. mutans* strains were evaluated. These included nine clinical isolates of *S. mutans* corresponding to distinct genotypes as defined in previous studies (21). These nine strains were previously characterized with respect to polymorphisms and amounts of GtfB, GtfC (19), and GbpB (17) accumulated in the culture fluids of 18-h planktonic cultures. Two of these strains (5ST1 and 3SN1) did not produce GtfB that could be detected by the GtfB-specific monoclonal antibody P72 (9). Strain UA159, whose genome is available (1), and its variant LT11 (31) (kindly provided by Dr Howard K. Kuramitsu, State University of New York at Buffalo, Buffalo, NY) were also included in this study. Strains were grown at 37°C (in 10% CO_2, 90% air) from frozen stocks in brain–heart infusion agar (Merck Labs, Darmstadt, Germany) for 24 h. Four colonies were then inoculated into 3 ml brain–heart infusion broth and grown for 18 h at 37°C (80% N_2, 10% CO_2, 10% H_2). Cultures (0.1 ml) were then adjusted to an absorbance at 550 nm ($A_{550\ nm}$) of 1, transferred to vessels containing 45 ml fresh prewarmed brain–heart infusion medium, and incubated from 8 to 18 h at 37°C in anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for determination of growth curves. For this purpose, culture samples were withdrawn at each 1- to 2-h interval for measurement of absorbance (A_{550nm}), and bacterial cells were collected by centrifugation, then frozen at −70°C for RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was purified from cells collected from 40 ml of culture at each time-point as described previously (24). RNA was then resuspended in diethyl pyrocarbonate-treated water (Invitrogen, Carlsbad, CA). RNA sample concentration and integrity were determined spectrophotometrically at 260 nm and in 1.2% formaldehyde agarose gels stained with ethidium bromide. Samples were then treated with DNase I (Invitrogen) under the conditions recommended by the manufacturer.

Reverse transcription reactions were performed with 12 ng total RNA using arbitrary primers Ea1 (TTTTATCCAGC), Ea7 (TCTTTTTTACC), Es1 (GCTGGAA AAA), Es3 (GAAGTGCTGG), and Es8 (TGCCGATGAA) (4), using the Superscript III system (Invitrogen) as recommended by the manufacturer. Briefly, RNA samples were preheated at 65°C for 5 min, and then added to reaction mixtures containing 2 μM for each primer. Reverse transcription took place in the following cycle: 25°C for 10 min, 50°C for 50 min, and 85°C for 5 min for enzyme denaturation. Reaction mixtures were then stored at −20°C. Negative controls for cDNA synthesis included samples with no RNA template, and sample reactions without reverse transcriptase.

Gene expression analysis by semi-quantitative reverse transcription–polymerase chain reaction

The semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of gene expression was based on a method that is described elsewhere (23). For this purpose, primers specific to each gene were designed based on the genome of strain UA159 (1) to generate amplicons ranging from 150 to 200 base pairs (Table 1). Curves for amplicons per number of thermal cycles specific for each primer were then generated to identify the middle of exponential phase of amplification. Briefly, PCR amplification took place in a final volume of 25 μl, which contained 18.125 μl H_2O milli-Q, 2.5 μl PCR 10× Buffer, 0.5 μl dNTP mixture 10 mM, 1.25 μl $MgCl_2$ 50 mM, 0.75 μl forward primer 10 mM, 0.75 μl reverse primer 10 mM and 0.125 μl Taq DNA 5 U/μl (Invitrogen), prepared as the master mix. Then, 1 μl of each cDNA sample was individually added. The thermal cycling parameters were as follows: 94°C for 45 s, annealing temperature (Table 1) for 60 s, and 72°C for 60 s, with variable cycle number from 15 to 60 for each pair of specific primers. PCR products were electrophoretically resolved in 2% agarose gels (0.15 μg ethidium bromide per ml) and digital images were captured under ultraviolet light with the Gel logic 100 Imaging System (Eastman Kodak Co., Rochester, NY).

Amplicon amounts were quantified densitometrically using the same imaging system and each cycle number was plotted to express the number corresponding to the middle of the exponential curve of amplification for each target gene. The selected numbers of cycles for each gene (Table 1) were then used for the semi-quantitative RT-PCR analysis with all cDNA samples, as previously described (23). Negative controls for PCR included reactions without template; positive controls included genomic DNA. Intensities of amplicons observed in the standard gels were expressed as arbitrary units of expression (U).

Four genes were tested as candidates for reference genes *16S* (38), *ldh* (20), *recA* (7), and *gyrA* (13). Table 2 shows mean and median values of the sum of transcription levels obtained within the entire growth curve obtained for each of the 11 strains analyzed. Transcription levels of each gene were assessed from a single pool of cDNA obtained from a particular culture sample. Five time-points were chosen to define five specific phases of

Table 1. Primer sequence, amplicon size, and thermal conditions applied in the semi-quantitative reverse transcription polymerase chain reactions

Gene	Gene ID[1]	Primer sequence (5′ → 3′)		Product size (bp)	Number of cycles	Annealing temperature (°C)
16SRNA	2886106	Forward	CGGCAAGCTAATCTCTGAAA	190	26	54
		Reverse	GCCCCTAAAAGGTTACCTCA			
ldh	1028428	Forward	TGGCGAAGAATTGATTGACT	183	40	54
		Reverse	TTGAAACCATATTGGCCATC			
recA	1029255	Forward	AAAGCTGCCACTGAGTCAAC	173	40	54
		Reverse	TTCATTGATGCAGAACATGC			
gyrA[2]	1028427	Forward	ATTGTTGCTCGGGCTCTTCCAG	105	40	58
		Reverse	ATGCGGCTTGTCAGGAGTAACC			
gtfB	1028336	Forward	CGAAATCCCAAATTTCTAATGA	197	40	54
		Reverse	TGTTTCCCCAACAGTATAAGGA			
gtfC	1028343	Forward	ACCAACCGCCACTGTTACT	161	40	54
		Reverse	AACGGTTTACCGCTTTTGAT			
gbpB	1029610	Forward	CAACAGAAGCACAACCATCA	151	40	54
		Reverse	TGTCCACCATTACCCCAGT			
comE	1029114	Forward	ATTGACGCTATCCCTGAAAAG	170	40	54
		Reverse	TGAAAAGTGAGGGGCATAAA			
ciaR	1028441	Forward	GAAGCAGAGAGTGGCGTTTA	156	40	54
		Reverse	ACCGTGTCCTTTGTCATCC			
covR	1029123	Forward	ACGAAATATGGCACGAACAC	185	40	54
		Reverse	CAGAGATGGACGGGTATGAA			
vicR	1028759	Forward	AGTGGCTGAGGAAAATGCTT	163	40	54
		Reverse	CATCACCTGACCTGTGTGTG			
rr11	1029476	Forward	TGGTTCGATTGGGTTTGA	161	40	57
		Reverse	GGCTTCTACACCATCCATTTC			

[1]GenBank: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene.
[2]Primer sequence from (13).

Table 2. Absolute values of expression of *16SRNA, ldh*, *recA*, and *gyrA* within the growth phases of 11 *Streptococcus mutans* strains

	16SRNA			*ldh*			*recA*			*gyrA*		
Strain	Mean[1]	Median	± SD (%[2])	Mean[1]	Median	± SD (%[2])	Mean[1]	Median	± SD (%[2])	Mean[1]	Median	± SD (%[2])
3VF2[3]	24.33	24.51	1.0 (3.9)	18.34	20.08	3.9 (21.9)	13.28	12.17	3.2 (24.4)	19.65	18.16	3.2 (16.3)
2ST1	24.24	23.65	2.2 (9.2)	21.68	21.07	2.4 (11.2)	18.28	17.98	3.0 (16.2)	19.58	19.57	2.2 (11.1)
8A1	22.27	23.80	2.7 (11.9)	19.67	20.16	4.4 (22.4)	17.75	19.78	5.4 (30.5)	22.30	22.27	4.0 (17.9)
1ID3	22.38	22.53	1.8 (7.9)	21.45	21.49	4.6 (21.6)	17.84	17.70	4.3 (23.9)	22.41	22.34	2.6 (11.4)
4JP2	24.78	25.76	1.9 (7.7)	24.62	25.20	2.5 (10.0)	21.32	21.41	2.5 (11.9)	22.05	22.44	2.9 (13.2)
5SM4	25.24	25.38	1.2 (4.8)	22.41	21.83	1.4 (6.5)	20.74	19.60	1.9 (9.0)	21.98	22.74	2.7 (12.4)
3SN1	21.37	19.76	2.8 (12.9)	18.71	16.75	4.7 (25.1)	17.12	17.15	3.8 (22.2)	19.83	21.83	3.3 (16.6)
5ST1	21.16	21.18	1.7 (7.9)	19.94	20.68	1.9 (9.6)	11.97	11.40	3.0 (25.1)	20.71	22.48	5.3 (25.4)
20A3	22.95	23.5	1.50 (6.5)	20.96	20.9	2.07 (9.9)	18.83	19.3	3.08 (16.4)	21.32	20.18	3.5 (16.2)
UA159[3]	24.91	25.10	0.9 (3.7)	11.77	11.52	2.0 (16.6)	16.33	16.81	6.7 (40.9)	20.61	20.51	2.0 (9.8)
LT11	20.15	20.97	1.2 (6.1)	18.20	17.82	2.7 (15.1)	16.15	16.38	3.7 (23.1)	24.86	25.35	3.6 (14.7)
Mean			(7.50)[4]			(15.45)[4]			(22.15)[4]			(15.00)[4]

[1]Mean of absolute units of expression measured in five time-points representative of each phase of growth.
[2]Percent proportion of the SD in relation to mean values.
[3]Only four time-points were analyzed.
[4]Mean of the percentage values of the SD in relation to mean values.

growth for the strains analyzed. However, for two strains, UA159 and 3VF2, only four time-points were chosen because early-log and late-stationary samples could not be assessed from their respective growth curves. Each time-point was within a range of culture absorbance ($A_{550\ nm}$) observed for the respective strain analyzed, except for strain 5SM4, which showed a low growth yield (see below). The early-log phase corresponded to the earliest time-point of the exponential phase of growth and was below the midpoint of the slope of each growth curve ($A_{550\ nm} <$ 0.33; mean: 0.24 ± 0.05). The mid-log sample corresponded to the time-point closest to the mid-point of the exponential phase ($A_{550\ nm}$ range: 0.37–0.63; mean: 0.47 ± 0.09). The late-log time-point corresponded to the time-point closest to the end of the exponential phase of growth ($A_{550\ nm}$ range: 0.67–0.84, mean.78 ± 0.05). The early-stationary phase time-point corresponded to the first time-point following plateauing of the growth curve slope (always observed after 14–16 h of growth). The late-stationary phase time-point was taken as the last time-point observed within the stationary phase (generally obtained at 18 h of growth). For 5SM4 the five growth phases corresponded to $A_{550\ nm}$ 0.21, 0.31, 0.40, 0.52, and 0.53 respectively.

Standard deviation values (SD) for *16S* reflected relatively small percentages (generally less than 10%; mean 7.5%) of the mean values of expression obtained during the growth curves, among all the strains tested. However, *ldh*, *gyrA* and *recA* replicates were more variable within the growth curves of the tested strains, with SDs ranging up to 25.1% of mean value for *ldh*, 25.4% for *gyrA* and 40.9% for *recA. 16S* was also less variable between strains in comparison with other reference genes.

Thus *16S* was selected for virulence gene normalization. Values of U of the target genes normalized by the U of *16S* were expressed as relative units of expression (RU).

Determinations of transcript levels of virulence genes were performed in two or three independent growth curve experiments; one to three time-points for each phase of growth were analyzed. From these initial screenings three different types of *gtfB* and *gtfC* curves of expression were identified. Strains that were representative of these three patterns of expression were then analyzed in three independent growth curve experiments performed on different days with different initial inocula. Data were then expressed as the mean of the three experiments. Variations in the levels of virulence and regulatory gene transcripts at each growth phase were tested using Kruskal–Wallis analysis. Comparisons between transcript levels of virulence genes were also performed for specific strains using the Mann–Whitney *U*-test, to verify gene coordination. Similar statistical analyses were also performed to compare transcript amounts of distinct *rr*.

Sequencing of *gtfB* upstream analysis

Strains 3SN1 and 5ST1 had been previously described as not showing detectable levels of expressed GtfB in immunoassays (19). The upstream *gtfB* region of these strains was sequenced to check for polymorphisms in their *gtfB* promoters. Briefly, *S. mutans* chromosomal DNA was isolated using a MasterPure DNA purification kit (Epicentre Technologies, Madison, WI) as described by the manufacturer. PCR products obtained with primers 5′-AGCGACAATTGTGGTGGGTA-3′ and 5′-TAACCAAGCCACCCGAAAGT-3′, were purified using a Stratagene PCR Purification Kit (Stratagene, La Jolla, CA), as specified by the manufacturer, and sequenced using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) at the Centro de Energia Nuclear, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo (CENA/ESALQ-USP, São Paulo, Brazil).

Results

Expression analysis of *gtfB*, *gtfC*, and *gbpB* genes

Expression of *gtfB* and *gtfC* was growth phase-dependent, and the highest value of RU divided by the lowest value of RU observed within a growth curve revealed a 1.5- to 6.2-fold increase or decrease in expression. Among the 11 strains analyzed, three distinct patterns of expression of *gtfB* and *gtfC* were identified: (i) coordinated expression of *gtfB* and *gtfC* during the entire growth curve, (ii) coordination of *gtfB* and *gtfC* expression in the log phase of growth but not at the stationary phase, and (iii) divergent expression of *gtfB* and *gtfC* (*gtfB/C*) both at log and stationary phases of growth. These patterns were almost equally prevalent among the strains tested, being found in four, four, and three of the strains, respectively. Figure 1A shows curves of *gtfB* and *gtfC* transcripts in strains that were representative of these three patterns of *gtfB/C* expression. Significant differences in *gtfB* and *gtfC* transcript levels (Mann–Whitney; $P < 0.01$) at specific phases of growth suggest independent regulation of these genes. Unexpectedly, *gtfB* transcripts were also detected in strains 3SN1 (shown in Fig. 1A) and 5ST1. Consistent with these results, these strains did not show mutations in their *gtfB* promoter regions, which were identical to those in strain UA159.

With one exception (Fig. 1A; strain 3VF2) transcript levels of *gbpB* showed only modest fluctuations in relation to growth phase. Curves of transcripts of *gbpB* were not coordinated with *gtf* genes (Fig. 1A).

Expression analysis of regulatory genes

Significant variations in the levels of each *rr* transcript at each phase of growth were observed among the 11 strains analyzed (Table 3). Patterns of *rr* gene expression were also strain-specific (Fig. 1B). For example, curves of expression of *covR* showed opposite patterns when strains 3SN1 and UA159 were compared (Fig. 1B). Curves of expression of *vicR* and *comE* were frequently coincident (Fig. 1B). Patterns of expression of *vicR* and *comE* genes were also coincident with other *rr*, such as *ciaR* and *rr11*, in several strains (data not shown). Expression of all response regulators was detected in most of the strains, except for *comE*, which was not expressed (or was expressed at very low levels) by the slow growing 5SM4 strain (Fig. 2). This strain carried the *comE* gene, as verified in PCR performed with genomic DNA, which showed that amplicons of expected size were generated with a similar yield to those observed for other studied strains (data not shown). Levels of *gtf* and *gbpB* transcripts in 5SM4 were similar to those observed in other strains (data not shown).

Discussion

GtfB, GtfC, and GbpB are important virulence factors of *S. mutans*, but the mechanisms that regulate their expression are still unclear. Several genetic studies have indicated that the genes involved in biofilm growth may be part of regulons under the control of distinct two-component transduction systems (3, 14, 15, 25, 27). Two of these systems, the inducer of virulence genes Vic (for virulence control), and the repressor of virulence genes Cov (for control of virulence), have been more recently associated with expression of *gbpB*, *gtfB*, and *gtfC* (3, 24). However, the genetic studies of *gtf* and *gbpB* regulation were performed within a context of only one or a few laboratory strains (3, 14, 15, 25, 27). Data on the diversity of expression of the Gtf-encoding and Gbp-encoding genes are scarce, and to our knowledge, this is the first study that explores the diversity of their patterns of expression in *S. mutans*.

Previously it was shown that differences in caries experience in *S. mutans*-infected children correlated with the differences in the capacities of the infecting strain to synthesize water-insoluble glucan (21). Thus *S. mutans* strains appear to differ with regard to their Gtf-mediated virulence. More recently, we showed that the amounts of GtfB, GtfC, and GbpB that accumulated in planktonic culture fluids after 18 h of incubation were associated with the respective strain's ability to grow as a biofilm *in vitro* (17, 19). In the present study, we analyzed gene expression patterns under planktonic conditions at specific phases of growth, because these conditions show relative physiological homogeneity. Such conditions may be more appropriate for strain comparisons because it has been reported that profiles of gene expression are variable within distinct areas/cell groups within the same biofilm (5). Such biofilm heterogeneity could bias interstrain comparisons.

A major aim when comparing patterns of gene expression within planktonic growth curves was to identify a reference gene that would be invariant within distinct phases of grow under the tested experimental conditions in each of the 11 strains compared. Three distinct reference genes that were previously used in analysis of *gtfB*, *gtfC* (7, 38), and *gbpB* (20) expression were evaluated in all the strains analyzed, and, of these, *16S* was shown to be the most suitable invariable gene (Table 2). This agrees with previous studies in *Staphylococcus aureus*, in which

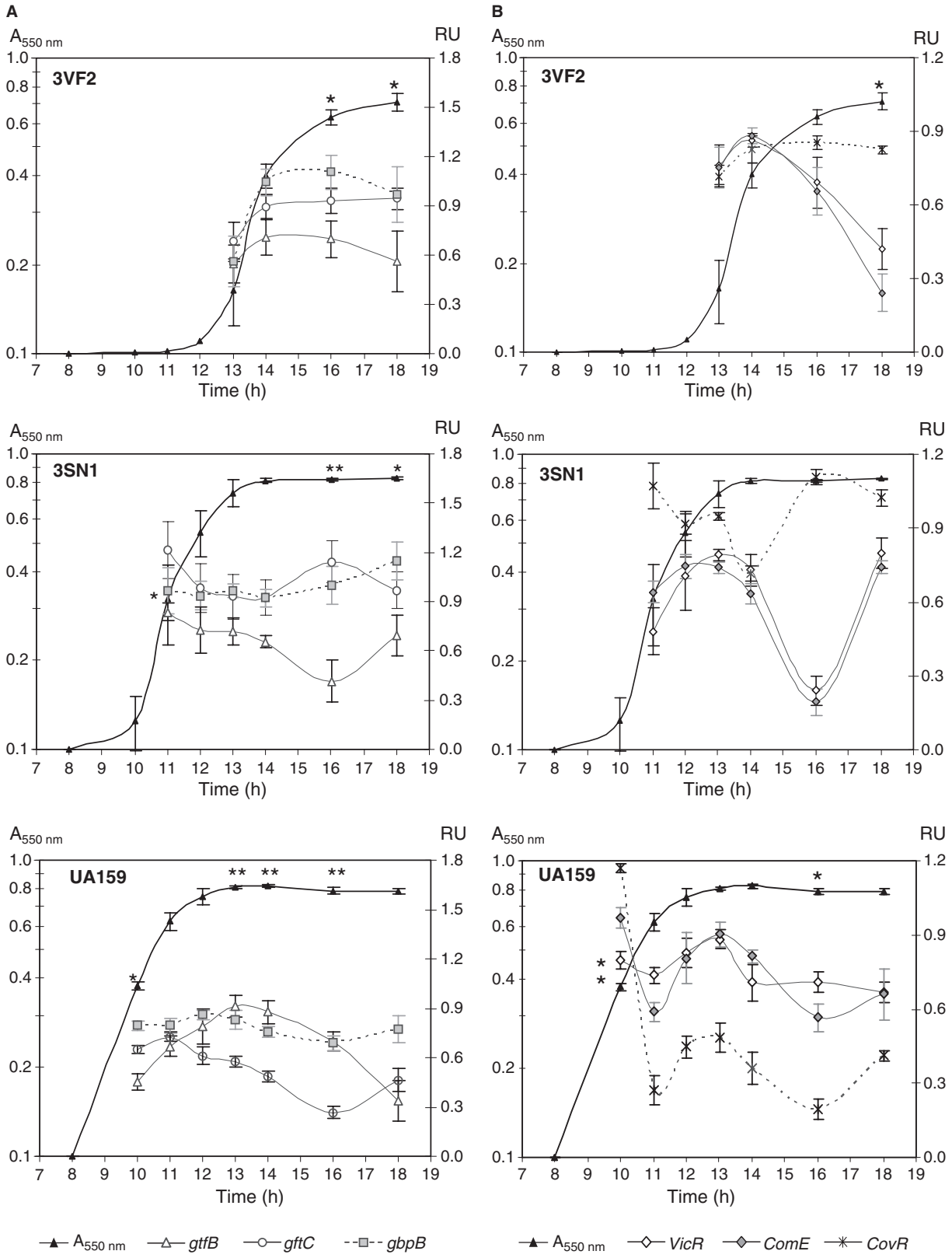

Fig. 1. Relative units of expression (RU, right scale) of virulence (A) and regulatory (B) genes, during different phases of planktonic growth ($A_{550\ nm}$, left scale), in three strains representative of the 11 isolates tested. (A) Transcription patterns of *gtfB*, *gtfC*, and *gbpB*. From top, strain 3VF2 showing *gtfB/C* gene coordination at log and stationary phases of growth, strain 3SN1 showing coordination between *gtfB/C* transcription only at log-phase, and strain UA159 showing divergent transcript levels of *gtfB*/*C* at both log and stationary phases of growth. (B) Patterns of *vicR*, *covR*, and *comE* in the respective strains. Results were reported as mean values of three independent experiments; error bars indicate standard deviation. Significant differences between levels of *gtfB* and *gtfC* transcripts (A), and between levels of *vicR* and *comE* transcripts (B) are indicated by asterisks at each respective point of the growth curve (Mann–Whitney *U*-test, $*P < 0.05$; $**P < 0.01$).

Table 3. Transcript levels of genes encoding response regulators (*rr*) in 11 clinical isolates at distinct phases of planktonic growth

Regulatory gene (*rr*)	Relative units of expression of *rr* in different phases of growth in 11 clinical isolates[1]				
	Early log[2] range (mean ± SD)	Mid-log range (mean ± SD)	Late-log range (mean ± SD)	Early-stationary range (mean ± SD)	Late-stationary[2] range (mean ± SD)
vicR	0.3–103.5 (58.2 ± 32.1)	17.6–93.7 (65.9 ± 26.3)	41.9–89.4 (80.8 ± 13.1)	23.7–103.5 (69.6 ± 27.4)	23.7–85.8 (55.9 ± 19.8)
covR	50.4–120.1 (90.5 ± 25.6)	23.0–118.7 (83.1 ± 29.5)	41.7–107.3 (81.4 ± 20.7)	16.8–116.7 (79.4 ± 27.4)	39.9–108.6 (81.0 ± 21.1)
comE	0.0–102.4 (59.6 ± 32.5)	0.1–82.1 (57.5 ± 23.9)	1.6–85.7 (62.6 ± 24.0)	0.5–88.6 (48.9 ± 32.1)	0.8–71.7 (42.4 ± 20.4)
rr11	3.7–45.5 (32.4 ± 13.0)	22.8–58.5 (36.6 ± 12.0)	20.0–77.9 (40.7 ± 18.0)	4.8–53.5 (30.8 ± 17.0)	5.7–42.9 (24.4 ± 10.0)
ciaR	16.7–89.9 (63.9 ± 19.0)	47.6–94.6 (64.5 ± 19.0)	56.5–84.2 (73.9 ± 9.0)	28.8–99.7 (64.4 ± 21.0)	30.1–92.2 (57.2 ± 19.0)

[1]Values were representative of two independent experiments, and multiplied by 100.
[2]Transcript levels were measured in only 10 strains.

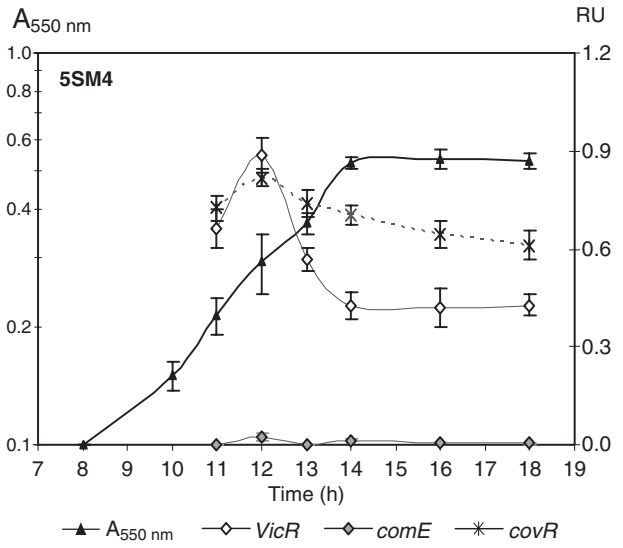

Fig. 2. Transcription levels of *rr* genes (RU, right scale) in strain 5SM4, which showed slow growth ($A_{550\ nm}$, left scale). Results were reported as mean values of three independent experiments; error bars indicate standard deviation.

putative reference genes (including *16S* and *gyrA*) were tested during different phases of planktonic growth (6). In that study, *16S* was also shown to be the most reliable standard gene. Gene *gyrA* has been successfully applied as a reference gene to identify genes involved in biofilm formation of *S. mutans* (Table 2), but *gyrA* was more variable than *16S* at different phases of growth (13). In *E. coli*, transcription of *gyrA* and *gyrB* were shown to be significantly affected by growth phase, apparently because DNA topology is involved in the coordination of changes in physiology/gene expression during growth (26).

The genes *gtfB* and *gtfC* are organized in an operon-like fashion in the *S. mutans* genome (35, 38). Previous studies exploring mechanisms of regulation of *gtfB* and *gtfC* have applied gene reporter fusions to the promoter region upstream to *gtfB*, thus assuming that both genes were part of an operon. However, a clear divergence in transcription levels of *gtfB* and *gtfC* (Fig. 1A) was detected in several strains. Alternatively, it is possible that the divergences observed in levels of *gtfB* and *gtfC* transcripts are the result of differences in RNA stability. However, given the high homology of these genes, it is more likely that variations in their transcript levels are a result of differences in gene activities rather than in the stability of the transcripts. Independent regulation of *gtfB* and *gtfC* was suggested in some studies (7, 10, 30, 37), but there are conflicting data in this regard (38) which may stem, in part, from differences in strain background. Independent patterns of *gtfB* and *gtfC* observed in some strains also might have implications for our understanding of the mechanisms of transcriptional regulation. For example, examination of the curves of the putative repressor *covR* (Fig. 1B) with curves of *gtfB* and *gtfC* transcripts (Fig. 1A) in strain 3SN1 suggested that *covR* could play a role in repressing *gtfB*, but not *gtfC*. Further studies will be necessary to analyze associations of specific regulators with the patterns of virulence gene expression in these strains. Additionally, little is known about the posttranscriptional regulation of these virulence genes.

Apart from distinct patterns of *gtf* expression, all the strains analyzed have shown activities of both genes *gtfB* and *gtfC*, consistent with previous evidence that both Gtfs play important roles in virulence (33, 36). Even strains 5ST1 and 3SN1, which had been previously reported to have undetectable levels of GtfB in culture fluids (19), showed *gtfB* transcripts. The latter observation agrees with the absence of polymorphisms at the *gtfB* promoter region in these strains. As previously shown using different culture conditions (19), GtfB produced by these strains in the present study was not recognized by the GtfB-specific monoclonal antibody MAb P72 (data not shown). We hypothesize that these proteins may be polymorphic at the MAb P72 epitope. Several polymorphisms have been identified in *gtfB* genes among distinct strains (8). Polymorphisms at the gene sequences targeted in the present study could influence levels of gene transcripts through primer mismatches. However, the efficiencies of the pairs of specific primers applied in the present study were quite similar between strains, as verified in PCR using genomic DNA as template (data not shown).

As for *gtf* genes, all the strains also expressed *gbpB*. However, little growth-phase variation was detected in *gbp*B transcript levels when compared to *gtf* genes (Fig. 1A). GbpB appears to play an essential function in *S. mutans* viability (20). This protein has also been described as a stress-response protein (4), but induction of *gbpB* expression by stress conditions appears also to be a strain-dependent phenomenon (20).

Differences in the patterns of *gtfB/C* and *gbpB* transcription observed between the strains analyzed could be the result of absence and/or different patterns of expression of transcriptional regulators that were previously demonstrated to affect expression of these virulence genes or biofilm

formation (1, 3, 11, 14, 15, 25, 27). Significant variation in transcription levels of several virulence genes (including *gtfB* and *gtfC*) among the wild-type Xc100L *S. mutans* strain and its subcultured variant Xc strain, was not associated with promoter polymorphisms, suggesting that specific factor(s) might have an influence on transcriptional activities of multiple virulence genes (37). All the strains expressed each of the *rr* analyzed, except for strain 5SM4. Strain 5SM4 did not express *comE*, and showed slow growth (Fig. 2), but it did express *gtfB/C* and *gbpB* transcripts at levels that were comparable to those in other strains expressing this *rr* (data not shown). Transcriptional levels of all *rr* varied widely between strains for each growth phase analyzed (Table 3), and patterns of expression in growth curves were highly variable (Fig. 1B). These differences might be a result of broader genomic diversity among the strains analyzed. Extensive genetic diversity involving several chromosomal deletions and/or insertions have been described among wild-type *S. mutans* strains (34). The strains analyzed in this study were selected from a set of 80 isolates that infected children at an early age (21), and that accounted for a total of 44 distinct arbitrarily primed PCR genotypic patterns (18). Several genomic loci have been characterized in these strains including the *gtfB/C* (19), *gbpB* (20) and the competence gene loci (12), providing evidence that they were representative of the *S. mutans* species.

Growth-phase-induced environmental signals might reflect changes in population density, cell cycle, and stress conditions related to the production of metabolic byproducts and nutrient availability. Expression of genes involved in cell damage and survival, for example, bacteriocin production, have been reported to be elevated in the stationary growth phase (24). Some of the bacteriocin-encoding genes are apparently regulated by the *rr* genes analyzed in *S. mutans* (25). In the present study, the same growth condition was applied for comparisons of all the strains. An interesting finding was that in the majority of strains, several *rr* were expressed in a highly coincident manner, as exemplified by the curves of transcripts of *vicR* and *comE* (Fig. 1B). This coordinated expression presents challenges in the assessment of individual effects of *rr* genes in the expression of *gtf* and *gbpB* genes (3, 27). Different growth conditions might also influence patterns of virulence and regulatory gene expression (28). For example, the Vic system contains a PAS domain at the VicK histidine kinase (27) that is conserved among proteins affected by changes in oxygen and redox potential (32). Fluctuations in transcript levels of *vicR* were observed within the growth curves of several strains, in several cases coincidently with other *rr* genes, as exemplified in Fig. 2, despite the growth under anaerobic conditions. Further studies are necessary to elucidate the environmental signals affecting Vic and other two-component transduction systems involved in the regulation of virulence genes. The strain-specific patterns of *gtf*, *gbpB*, and *rr* genes revealed in the present study indicate that it is important to consider inter-strain variation when exploring genetic mechanisms that control virulence genes. This may have further implications in the selection of therapeutic targets to control *S. mutans* infection.

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