Oral Streptococci with Genetic Determinants Similar to the Glucosyltransferase Regulatory Gene, *rgg*

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Received 15 May 1995/Returned for modification 19 July 1995/Accepted 23 August 1995

The *Streptococcus gordonii* Challis glucosyltransferase structural gene, *gtfG*, is positively regulated by the upstream gene, *rgg*, the only described *gtf* regulatory determinant in oral streptococci. Southern hybridization analyses indicated that *rgg*-like and *gtfG*-like determinants were present on the same *Hind*III fragment in strains of *S. gordonii*, *Streptococcus sanguis*, and *Streptococcus oralis*, whereas no *rgg*-like determinants were detected in mutans streptococci, *Streptococcus mitis*, and *Streptococcus salivarius*.

Many oral streptococci have glucosyltransferase (GTF) enzymes which hydrolyze sucrose and polymerize the glucose moiety to form water-soluble and/or water-insoluble glucans. The GTF enzymes, as well as the glucans they form, may play important roles in the development of dental plaque. The glucan polymers have been implicated in promoting the adhesion, aggregation, and accumulation of several streptococcal species on the tooth surface, which thereby influence plaque composition (7). The streptococcal glucans have been studied most extensively because of their potential as virulence factors for the mutans streptococci in dental caries (18). In addition, these polymers may play other roles in the oral cavity, such as modulating the diffusion of substances through plaque (20, 31) and possibly serving as extracellular energy reserves (2).

Despite the significant ecological roles of GTF enzymes and their glucan products, little is known about their regulation. The mutans streptococci and *Streptococcus salivarius* have multiple GTF enzymes coded for by multiple *gtf* genes (3, 8, 9, 12, 24, 25, 30). No genetic regulatory mechanisms for these *gtf* genes have been described. In contrast to the mutans streptococci and *S. salivarius*, *Streptococcus gordonii* Challis CH1 has a single GTF enzyme coded for by the structural gene, *gtfG* (28). Furthermore, an important *gtf* regulatory determinant, designated *rgg*, has been identified in this strain. *rgg* is the only regulatory determinant for a *gtf* gene described for oral streptococci. Genetic studies have shown that *rgg*, located immediately upstream of the *gtfG* structural gene, encodes a positive activator of *gtfG* (28). Nucleotide sequence analysis suggests that *rgg* encodes a ca. 34.5-kDa cytoplasmic protein. The *rgg* open reading frame is preceded by a putative promoter followed by a region of dyad symmetry, suggestive of a possible regulatory factor binding site, and a putative ribosome binding site. *gtfG* has its own putative promoter and ribosomal binding site located within an inverted repeat. Mutants with a premature translational stop site in *rgg* have significantly decreased GTF activity; Northern (RNA) blot analyses show that these mutants make less *gtfG*-specific transcript (27). Transformation of these mutant strains with plasmids carrying a functional

rgg results in increased levels of the *gtfG*-specific transcript and GTF activity. Although the basis of regulation of *gtfG* by *rgg* is unknown, the data are consistent with a model in which the Rgg protein acts at the *gtfG* promoter to increase *gtfG* transcription. Nucleotide sequence analysis also suggests that *gtfG* expression may be affected at another level. A stem-loop corresponding to the transcription terminator of *rgg* but containing the *gtfG* ribosome binding site may form in the mRNA and influence the efficiency of *gtfG* translation. Such translational coupling has been described in *Escherichia coli* (10), and its implications for *gtfG* regulation have been discussed previously (28). Alternatively, distally located genes may affect expression of *rgg* and/or *gtfG*.

No genes with significant overall nucleotide or amino acid homology to *rgg* have been reported for any other oral streptococci in GenBank or EMBL sequence data banks. However, a gene similar to *rgg*, but of unknown function, has been identified recently in *Lactococcus lactis* (6). The finding of an *rgg*-like determinant *L. lactis*, which has no GTF activity, raises the intriguing possibility that *rgg* may share characteristics with other regulatory elements in gram-positive bacteria.

Since *rgg* is the first characterized regulatory gene for a *gtf* determinant and has been described only in *S. gordonii* Challis, Southern hybridization analyses (26) were done to determine if *rgg*-like determinants were present in other strains of oral streptococci. Thirty strains representing various species of oral streptococci were examined (Table 1). Strains were chosen to represent the three biovars of *S. gordonii* and the four biovars of *Streptococcus sanguis* (16). Well-characterized strains representing each of the mutans streptococcal species, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus downei*, *Streptococcus rattus*, and *Streptococcus cricetus*, were included. Strains of *Streptococcus oralis*, which was previously classified as *S. sanguis* (16), as well as *S. salivarius*, which has at least five *gtf* genes (25), were also included. Culture supernatants of all these strains gave rise to a glucan band(s) or sodium dodecyl sulfate (SDS)-polyacrylamide gels, which represented GTF activity (22; data not shown). In addition to these oral streptococci with known GTF activity, two strains of *Streptococcus mitis* were included in this study. Although this species has no reported GTF activity (16) and no bands were seen for the strains examined with GTF activity gels (data not shown), the presence of *gtf*-like DNA sequences in *S. mitis* is possible, especially considering the genetic exchange that may occur in the oral environment (23). Furthermore, the finding of *rgg*-like genes in lactococci (discussed above) suggests that the function

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TABLE 1. Fragments hybridized to probes in Southern hybridization analyses of strains of oral streptococci

Species	Strain ^a	Fragment size (kb) ^b	
		<i>rgg</i> probe	<i>gtfG</i> probe
<i>S. gordonii</i>	CH1	4.0	4.0
	G9B	3.1	3.1
	ATCC 12396	3.1	3.1
	SK3	4.0	4.0
	SK86	4.0	4.0
	SK8	3.1, 2.3, 4.0	3.1, 2.3
	SK9	3.1	3.1
	SK186	3.1	3.1
	FC1	2.3	2.3
<i>S. sanguis</i>	ATCC 10556	2.3	2.3
	SK108	2.3	2.3
	SK115	2.3	2.3
	SK150	2.3, 4.4	2.3, 2.7
	SK45	2.3	2.3
	C5	4.0, 4.4	4.0, 4.4
<i>S. oralis</i>	ATCC 35037	5.6	5.6
	NCTC 7864	1.8, 0.5, 3.1	1.8
<i>S. mitis</i>	NS51	— ^c	—
	ATCC 903	—	—
<i>S. salivarius</i>	ATCC 25975	—	3.9, 1.5
<i>S. mutans</i>	Ingbritt	—	4.6, 2.9
	LM7	—	—
	ATCC 25175	—	4.6, 3.2, 1.8
	3209	—	—
	GS5	—	4.6, 2.9
	NCTC 10449	—	—
<i>S. sobrinus</i>	SL1	—	—
	6715	—	9.0
<i>S. downei</i>	MFe28	—	7.2
<i>S. rattus</i>	FA1	—	2.9
<i>S. cricetus</i>	AHT	—	3.3

^a CH1 is the Challis strain (29) from which *rgg* and *gtfG* were cloned. The strains tested were from the following laboratories: B. Rosan, Philadelphia, Pa. (G9B); American Type Culture Collection, Rockville, Md. (ATCC 12396, ATCC 10556, ATCC 903); M. Kilian, Aarhus, Denmark (SK3, SK86, SK8, SK9, SK186, SK108, SK115, SK150, SK45); R. Gibbons, Boston, Mass. (FC1, C5); J. Ferretti, Oklahoma City, Okla. (ATCC 35037, NCTC 7864, NS51, ATCC 25975, Ingbritt, ATCC 25175, 3209, GS5, SL1, 6715, MFe28, FA1, AHT); F. Macrina, Richmond, Va. (LM7); and R. R. B. Russell, Newcastle, United Kingdom (NCTC 10449).

^b Size in kilobases of the *Hind*III fragment from each strain that hybridized to the *rgg* or *gtfG* probe (Fig. 1). When multiple *Hind*III fragment sizes are listed, they are in the order of the intensity of the bands. All strains were tested with each probe separately at least twice. To confirm that the *rgg*-like and *gtfG*-like fragments were the same size in strains with fragments that hybridized to both probes, membranes were hybridized first with one probe, stripped, and then rehybridized with the second probe according to the manufacturer's directions. In each case, the bands were superimposable, regardless of the order of the probes used, indicating that the fragments that hybridized to the probes were as close to the same size as could be detected by our Southern analyses.

^c No fragments detected by probe.

of *rgg*, when it is present, may not be limited to the regulation of *gtf* determinants.

Bacteria were grown to the late log stage in Todd-Hewitt broth (Difco, Detroit, Mich.) containing 0.5% glycine. Chromosomal DNA from each strain was obtained by a modified (28) alkaline lysis method. The DNAs of *S. oralis* NCTC 7864 and *S. rattus* FA1 were treated with cetyltrimethylammonium bromide (1) to remove excessive carbohydrates and optimize digestion by restriction enzymes. Chromosomal DNA from each strain was digested with *Hind*III, electrophoresed on 0.7% agarose, and blotted to Hybond-N nylon membranes (Amersham). DNA probes for internal portions of the *rgg* and *gtfG* genes were made by PCR (Fig. 1). Template DNA was the

FIG. 1. Schematic diagram of the plasmid clone pAMS40 insert. The diagram shows the approximate positions of the *rgg* and *gtfG* open reading frames, indicated by the heavy arrows above the restriction digest map, and the 619-bp *rgg* and 1,563-bp *gtfG* probes used for Southern hybridization analyses. The probes had G + C contents of 37% (28) and 43% (34a), respectively.

plasmid clone pAMS40 (27), which contains the *S. gordonii* Challis *gtfG* gene and its flanking regions cloned into the *Hind*III site of the streptococcal vector pVA749 (19). To make the *rgg* probe, oligonucleotide primers 5'-CTGTTGCCAGC TGTC3' and 5'-CCGGTCATAGAGGTCT3' were used to amplify 619 bp of the internal region of *rgg*. The *gtfG* probe was designed to detect the region encoding approximately the amino-terminal one-third of the processed GTF protein. To increase specificity, the region encoding the signal sequence of *gtfG* was excluded from the *gtfG* probe, since regions encoding signal sequences can be conserved (12, 35) and a probe containing these nucleotides could potentially hybridize to genes encoding a number of exported streptococcal proteins. Primers 5'-GAATCAGGTGTGATCTATGC3' and 5'-AAGCTTCCAA GATAGACAG3' were used to amplify a 1,563-bp region of the pAMS40 template to make the *gtfG* probe. The resulting *rgg*- and *gtfG*-specific fragments were cleaned with a Qiaex PCR purification kit (Qiagen, Chatsworth, Calif.) and labelled with digoxigenin-dUTP by using the Genius system (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's directions. The probes were hybridized to the membranes at 68°C, and hybridization was followed by two room temperature washes in 0.1% SDS-2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.4) for 5 min each and two 15-min washes at 68°C in 0.1% SDS-0.1× SSC (pH 7.4) (1). Fragments that hybridized to the probes were detected by chemiluminescence. In some cases, the blots were stripped with a solution of 60% formamide and 1% SDS in 50 mM Tris-HCl (pH 8.0), checked to confirm the absence of probe, and then rehybridized with the second digoxigenin-dUTP-labelled probe according to the manufacturer's directions.

Thirteen of the strains examined, including representative strains of *S. mitis*, *S. mutans*, *S. sobrinus*, *S. downei*, *S. rattus*, *S. cricetus*, and *S. salivarius*, did not have determinants that hybridized to the *rgg* probe (Table 1). However, several, but not all, of the mutans streptococci and *S. salivarius* did have determinants that hybridized to the *gtfG* probe (Table 1). The *gtfG* probe contained (34a) the region encoding the conserved catalytic sequences thought to be involved in sucrose hydrolysis (5, 21); however, it did not include the region encoding the conserved signal sequence. Published data on the presently sequenced *gtf* genes indicate that the first ca. 600 bp after the region encoding the signal sequence of each *gtf* are unique for each gene (23). Thus, the variable extent of hybridization of the *gtfG* probe to chromosomal DNA of streptococcal strains known to have *gtf* genes is consistent with the known *gtf* nucleotide sequence data (3, 8, 9, 12, 23–25, 30).

The results of the Southern analyses with the *rgg* probe suggest that the *gtf* genes in these strains of mutans streptococci and *S. salivarius* are not regulated by an *rgg*-like gene. No

FIG. 2. Southern hybridization analyses of chromosomal DNA digested with *Hind*III. DNA size standards (Bethesda Research Laboratories, Gaithersburg, Md.) ran as indicated by the numbered marks on the right of each blot. *S. gordonii* Challis CH1 was run in the first lane of each gel as a control. Probe DNA was labelled with digoxigenin-dUTP, hybridized to the membrane under stringent conditions (1), and detected by chemiluminescence by using the Genius system (Boehringer Mannheim) according to the manufacturer's directions. For this illustration, the films were scanned directly, without enhancement, with an OmniMedia 6cx scanner (X-ray Scanner Corporation, Torrance, Calif.). The images were labelled with Adobe Illustrator, version 3.2, for Macintosh (Adobe Systems Inc., Mountain View, Calif.). (A) Strains of *S. gordonii* hybridized with the *rgg* probe. (B) Strains of *S. sanguis* and *S. oralis* hybridized with the *rgg* probe. (C) Strains of *S. gordonii* hybridized with the *gtfG* probe. (D) Strains of *S. sanguis* and *S. oralis* hybridized with the *gtfG* probe.

gtf regulatory genes have been identified in these species. Studies with reporter genes suggest that environmental conditions affect expression of *S. mutans gtf* (13, 36), but the basis of this regulation is unknown. Regulation of the GTF enzymes may occur at a posttranslational level; it has been hypothesized that regulation of the level of *S. salivarius* GTF activity may occur at the level of protein translocation across the cytoplasmic membrane (8). However, the existence of regulatory Rgg-like proteins coded for by genes that were not detected in the present study cannot be ruled out.

All strains of *S. gordonii*, *S. sanguis*, and *S. oralis* examined in this study had DNA sequences that hybridized to the *rgg* probe (Fig. 2A and B). Some strains had multiple *Hind*III fragments that hybridized to the *rgg* probe. These multiple bands may be due to *Hind*III sites within the *rgg*-like determinants in these strains that result in the hybridization of the probe to multiple fragments. This was supported by the observation that digestion of the chromosomal DNA of these strains with other restriction enzymes resulted in fewer fragments hybridizing to the *rgg* probe (data not shown). Additional fragments that hybridized weakly to the *rgg* probe, such as those in strain SK8, may also be due to additional *rgg*-like nucleotide sequences within the chromosome. Each strain that had an *rgg*-like de-

terminant also had a determinant that hybridized strongly to the probe for *gtfG* (Fig. 2C and D). Furthermore, the *rgg*- and *gtfG*-like determinants appeared to be on the same *Hind*III fragment (Table 1) as that in *S. gordonii* CH1 (Fig. 1). The strong degree of hybridization of each probe (Fig. 2) suggests that these nucleotide sequences are highly conserved among these bacterial species.

In addition to those of the oral streptococci, several other relevant strains were examined in this study. *Leuconostoc mesenteroides* ATCC 14935 (from the laboratory of H. S. Fogler, Ann Arbor, Mich.) was examined because this species has a dextransucrase which is similar to the GTF enzymes of the oral streptococci (5); no mechanisms of genetic regulation have been described for this enzyme. The DNA of this strain had no fragments that hybridized to either the *rgg* or *gtfG* probe, indicating that the region of the dextransucrase gene encoding approximately the amino-terminal one-third of the protein is not highly similar to that of *gtfG* and that there is no detectable *rgg*-like determinant. Two strains of enterococci which can exchange DNA with streptococci but which have no GTF activity were also examined. As expected, *Enterococcus faecalis* OG1X (14) and JH2-2 (15) did not have DNA sequences that hybridized to the *gtfG* probe. Moreover, *E. faecalis* OG1X did not have a determinant that hybridized to the *rgg* probe. However, strain JH2-2 had a weak but distinct 3.6-kb band that hybridized to the *rgg* probe (data not shown). The function of the *rgg*-like determinant in this strain is unknown, but its presence lends support to the possibility that *rgg* may have functions in addition to the regulation of *gtf* genes.

All the oral streptococci in this study that had *rgg*-like determinants were members of species previously classified as *S. sanguis*, and all represent relatively early colonizers of the tooth surface (4, 17). Although in vitro studies suggest that glucans may increase the accumulation of these species on saliva-coated hydroxyapatite surfaces (32–34), the in vivo role(s) of GTF in these species and the influence of their glucans on plaque development have not been fully examined. Further studies are necessary to define the possible interactions of the GTF enzymes and glucan products of these species with other components of the oral environment. The GTFs of these strains may be regulated by posttranslational modifications (11). However, the present studies strongly suggest that the *gtf* genes in these early colonizing species may be regulated at a DNA level by an *rgg*-like determinant. The Southern hybridization patterns in the representative strains tested showed the proximity of the *rgg*- and *gtfG*-like determinants and support the possibility that *rgg*-like regulatory genes may be located upstream of the *gtf* structural genes in strains of *S. gordonii* in addition to strain Challis as well as in *S. sanguis* and *S. oralis*. We can further speculate that the regulation of *gtf* genes in these species may therefore occur by transcriptional and translational mechanisms similar to those that are proposed for *S. gordonii* Challis. The widespread occurrence of *rgg*-like determinants among these species suggests that further studies of the *gtf* genes and their regulation may provide important insights into dental plaque composition and the oral ecology.

We thank those who provided the bacterial strains used in these studies.

This work was supported by NIDR grants DE10217 and DE11090.

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