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Rgg Is a Positive Transcriptional Regulator of the *Streptococcus gordonii gtfG* Gene

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The *Streptococcus gordonii* (Challis) glucosyltransferase-encoding determinant *gtfG* is regulated by the product of the adjacent gene *rgg*. Results of analyses described here showed that in both *S. gordonii* and *Escherichia coli* Rgg is a positive transcriptional regulator of glucosyltransferase expression. In addition, the transcriptional start sites of both *gtfG* and *rgg* were determined.

Glucosyltransferases (GTFs) are enzymes that polymerize the glucose moiety of sucrose to yield glucan. The enzymes are thought to be important for the mutans streptococci in sucrose-dependent bacterial accumulation on smooth tooth surfaces (9). The production of glucan by *Streptococcus gordonii* has been correlated with the accumulation of this organism on hydroxyapatite beads (24); thus, GTF may play a role in the colonization of *S. gordonii* on the tooth surface. Experimental evidence also suggests that this enzyme may be a virulence factor for *S. gordonii* in the pathogenesis of infective endocarditis (12, 16), although a recent report suggests that this is not the case for *S. gordonii* Challis, at least in a rat endocarditis model (26).

The complete nucleotide sequence of *S. gordonii* Challis *gtfG* was determined recently (25), and the corresponding protein had a mass of ca. 170 kDa. We previously identified a gene, *rgg*, located immediately upstream of *gtfG*, that functions as a positive regulator of GTF expression (21). Haisman and Jenkinson have also identified two distinct genetic regulatory loci, *gtf-20* and *gtf-30*, that appear to regulate GTF expression (4). Here, we report the results of Northern (RNA) blot and primer extension analyses of the *rgg-gtfG* locus, which show (i) transcripts that are expressed at the *rgg* and *gtfG* loci and where they are initiated and (ii) that the *rgg* product affects GTF expression at the level of transcription. In addition, our conclusions are corroborated by the demonstration of regulation of *gtfG* in *Escherichia coli* with a *gtfG'-lacZ* transcriptional reporter fusion.

A restriction endonuclease map of the *rgg-gtfG* region of the *S. gordonii* Challis chromosome, related probes used for Northern hybridization, and sizes and locations of transcripts detected are shown in Fig. 1A. Several mRNA transcripts were identified in the strain containing the streptococcal cloning vector pVA749 (Fig. 1B, lanes 4). A 1.7-kb band was identified with the upstream probe. A 1.1-kb band was identified exclusively with the *rgg* probe, a 5.4-kb band only was identified with the *gtfG* probe, and a 6.4-kb band was identified with both the *rgg* and *gtfG* probes. The data are consistent with the following three transcripts derived from the *rgg* and *gtfG* loci (Fig. 1A): (i) a 1.1-kb *rgg* transcript, large enough to contain the *rgg* open reading frame (ORF) of 891 nucleotides; (ii) a 5.4-kb *gtfG* transcript that is of a size sufficient to contain the entire structural determinant of the GTF (22, 25); and (iii) a 6.4-kb *rgg-gtfG* transcript that likely represents a transcript originating at the *rgg* promoter and terminating at the same site as the 5.4-kb *gtfG* transcript.

The Challis *rgg*-defective strain DS512 contains a frameshift mutation in *rgg* and exhibits extracellular GTF activity at levels that are ca. 3% of that of the wild type (21). Northern blots of the mutant strain DS512(pVA749) (Fig. 1B, lanes 2) were compared with those of the wild-type strain CH1(pVA749) (Fig. 1B, lanes 4). With the *gtfG* probe, the 5.4-kb transcript seen in the wild-type strain was absent in the *rgg* mutant strain. No differences in mRNA patterns between the two strains were observed when the upstream and the *rgg* probes were used. Therefore, the frameshift mutation in the *rgg* gene resulted in the specific loss of the 5.4-kb *gtfG* transcript.

It was shown previously that when strain DS512 contained *rgg* supplied in *trans* by plasmid pAM-S57, it exhibited GTF levels above those observed in the wild type (21); the intensities of the bands of specific mRNA transcripts were also higher than that observed for the wild type. The chimeric multicopy plasmid pAM-S57 is composed of pVA749 and a 1.5-kb *Hae*III DNA fragment (see the restriction sites in Fig. 1A) containing the *rgg* gene and 99 bp of the 5′ end of the *gtfG* gene (21). The *gtfG* probe identified a 2.4-, a very intense 5.4-, and a >9.5-kb band for DS512(pAM-S57), bands not observed for the *rgg* mutant strain (Fig. 1B, lanes 3 and 2, respectively). Indeed, the same transcription patterns observed for DS512(pAM-S57) were also seen for CH1(pAM-S57) with all three probes (Fig. 1B, lanes 5). The increased copy number of the *rgg* gene when present in DS512 and CH1 appears to have increased the level of *gtfG* mRNA expression above that of the wild type. This increased transcription may have led to increased readthrough of the transcription-termination site of the *gtfG* gene, which in turn may have led to the visualization of the extended transcript of >9.5 kb in size.

Although complementation of the *rgg* mutant increased the levels of the 5.4-kb transcript, the levels of the 1.7-kb and 6.4-kb transcripts remained unaltered. That is, two bands from the *rgg* mutant (Fig. 1B, lanes 2), the 1.7-kb band observed when the upstream probe was used and a 6.4-kb band seen when the *rgg* or *gtfG* probe was used, had intensities equal to those of the same-size bands seen for strain DS512 containing *rgg* on the multicopy vector pAM-S57 (Fig. 1B, lanes 3). It should be noted that levels of both the *rgg*-specific 1.1- and 2.4-kb transcript with the *rgg* probe for DS512(pAM-S57) were elevated relative to the levels observed for the same two transcripts from DS512(pVA749) (Fig. 1B, lanes 3). These latter

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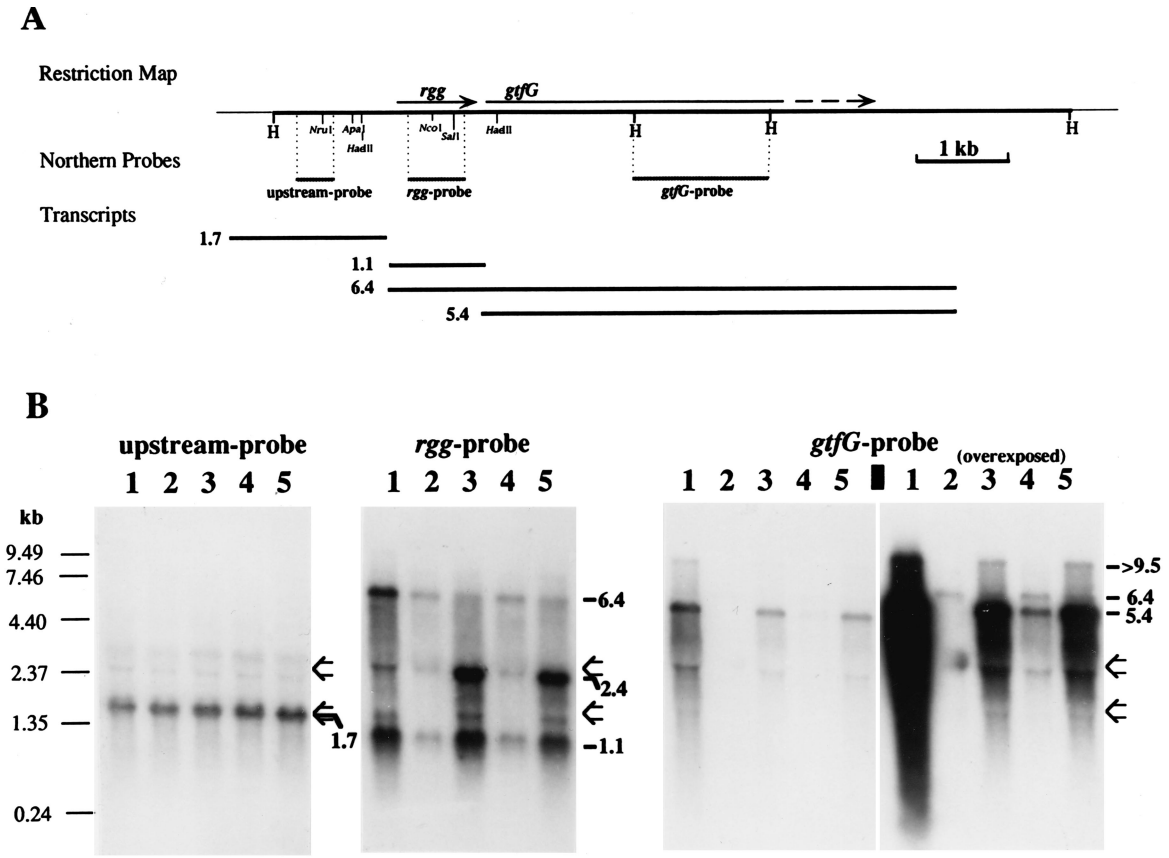

FIG. 1. Identification of transcripts at the *rgg-gtfG* locus by Northern blot analysis. (A) Schematic diagram depicting the approximate positions of DNA probes and mRNA transcripts, including approximate transcript sizes, in kilobases, as determined by the results of Northern blot analyses shown in panel B, relative to the restriction map of the *rgg-gtfG* locus (21). Arrows above the restriction map identify *rgg* and *gtfG* ORFs; broken lines at the end of the *gtfG* ORF arrow indicate that the *gtfG* ORF termination site is unknown. (B) Northern blots identifying transcripts. RNA from the following strains was analyzed: lanes 1, CH1(pAM-S40); lanes 2, DS512(pVA749); lanes 3, DS512(pAM-S57); lanes 4, CH1(pVA749); and lanes 5, CH1(pAM-S57). Northern blots were hybridized to the upstream probe (Northern blot on the left), the *rgg* probe (middle blot), and the *gtfG* probe (right blot) under stringent conditions. Sizes of the molecular size standards and hybridization bands, in kilobases, are shown. The positions of the 23S (top open arrows) and 16S (bottom open arrows) rRNA bands are indicated. Other investigators have observed that "shadows" in an mRNA pattern can be the result of rRNAs in the samples saturating the binding sites on the membranes. In addition, mRNAs were observed to accumulate in front of the rRNA band (6, 11). It is conceivable that the ca. 2.4- and ca. 1.4-kb bands seen in panel B, regardless of the probe used and excluding the 2.4-kb bands identified by the *rgg* probe in lanes 3 and 5, represent mRNAs that accumulated immediately ahead of the 23S rRNA and 16S rRNA, respectively. The 392-bp upstream probe DNA was synthesized by PCR with a GeneAmp PCR reagent kit and a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.). pAM-S13 (21) was used as the template DNA, and the two synthetic oligonucleotide primers 5′GTCCATGAATGGTGCT3′ and 5′AAAAGGCCAAGACCAC3′ were constructed (DNA Core Facility, University of Michigan, Ann Arbor) on the basis of sequence data obtained from the region upstream of the *rgg* gene (data not shown). The *rgg* probe DNA, prepared by PCR with pAM-S57 (21) as a template, and the oligonucleotide primers 5′CTGTTGCCCAGCTGTC3′ and 5′CCGGTCATAGA GGTCT3′ consisted of a 619-bp DNA segment internal to the *rgg* ORF. PCR products were purified by the Qiaex PCR purification procedure as described by the manufacturer (Qiagen, Inc., Chatsworth, Calif.). The *gtfG* probe was an internal *Hin*dIII fragment from pAM5010 (21) recovered from an agarose gel and subsequently purified by the Qiaex gel extraction procedure as described by the manufacturer. Total RNA was purified by a hot phenol method (17) from *S. gordonii* cells that were grown to mid-log phase (density with a Klett-Summerson colorimeter with a no. 54 filter, 65 Klett units) in Todd-Hewitt broth (Difco, Detroit, Mich.). RNA samples (6 μg; as judged by A_{260}) and RNA molecular weight markers (GIBCO BRL, Gaithersburg, Md.) were electrophoresed on a 1.0% agarose gel containing formaldehyde (1.1%) and transferred to nitrocellulose (BA-S NC; Schleicher & Schuell, Keene, N.H.). DNA was nick translated with [α-^{32}P]dATP (Amersham, Arlington Heights, Ill.) with a nick translation kit as directed by the manufacturer (GIBCO BRL). Hybridization under stringent conditions in a 50% formamide solution at 42°C, washing of the blot, and autoradiography were conducted as described elsewhere (1). Membranes were stripped of probes by the method of Sambrook et al. (13) to facilitate multiple hybridizations.

two bands likely represent transcripts from the *rgg* sequence present on the plasmid. Indeed, faint but distinct 2.4-kb transcripts were also seen in the strains containing pVA749. We, as well as others (6, 11), have noticed that the bands are artifacts of the Northern blot procedure (see also the legend to Fig. 1 for an explanation). The increased intensities of the 2.4-kb bands in strains containing pAM-S57 are explained as follows. Since the presence of the 6.4-kb transcript in the wild-type strain suggests that transcription of *rgg* can proceed into the *gtfG* gene and since pAM-S57 contains the *rgg* gene and the 5′ end of the *gtfG* gene, it is likely that transcripts from pAM-S57 include a 1.1-kb *rgg* transcript and a 2.4-kb extended *rgg* transcript that is transcribed to a termination site within the pVA749 portion of pAM-S57. Results of Northern analyses of RNA from CH1 containing pAM-S40, a chimeric plasmid consisting of pVA749 (vector) and the 8.8-kb fragment that contains the entire *rgg* and *gtfG* genes (20), lend support to this possibility. CH1(pAM-S40) was expected to synthesize a 6.4-kb *rgg* transcript with an intensity roughly equal to that seen for the 2.4-kb *rgg* transcript present in DS512(pAM-S57), since both are extended *rgg* transcripts from plasmids of equal copy numbers; this was indeed observed (Fig. 1B, *rgg* probe, lanes 1 and 3). Assuming the 1.1- and 2.4-kb *rgg* bands were due to enhanced transcription of *rgg* contained on the plasmid, we

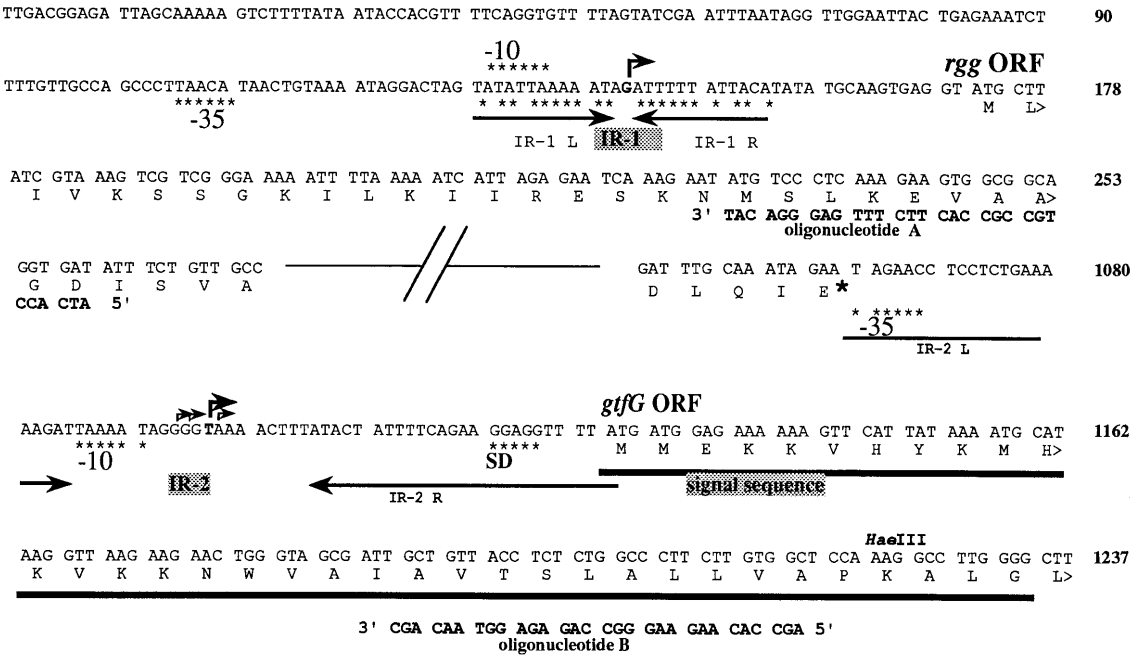

FIG. 2. Nucleotide and amino acid sequences of the *rgg* and *gtfG* promoter regions. The arrows above nucleotides 144 and 1097 denote *rgg* and *gtfG* transcriptional start sites obtained with oligonucleotide A and oligonucleotide B in primer extension reactions for *rgg* and *gtfG*, respectively (Fig. 3). Previously suggested −10 and −35 regions are shown and marked with small asterisks. Other features of this sequence were identified previously (21). The arms of two inverted repeats are indicated by arrows: (i) IR-1 R and IR-1 L and (ii) IR-2 R and IR-2 L. The putative signal sequence of GtfG is shown by the underlined 35 amino acids. An internal portion (774 bp) of the *rgg* ORF is not shown, and this omission is indicated by two slash marks. SD, Shine-Dalgarno sequence (marked with a large asterisk).

conclude that the *rgg* product was a *trans*-activator of transcripts exclusively identified with the *gtfG* probe.

Start sites identified by primer extension provided further information relating to transcription at these loci (Fig. 2 and 3). We used two 30-mer oligonucleotide primers complementary to sequences located near the 5′ ends of the *rgg* and *gtfG* genes (Fig. 2, oligonucleotides A and B, respectively). The apparent 5′ end of the *S. gordonii rgg* mRNA was a G residue (Fig. 3) located in the middle of the region of dyad symmetry at nucleotide 144 in Fig. 2; it is likely that initiation of both the *rgg* 1.1-kb and *rgg-gtfG* 6.4-kb transcripts occurred at this site. For the 5.4-kb *gtfG* mRNA the start site was not as definitive, with several alternative adjacent nucleotides being evident. The strongest band was that corresponding to a T residue located between the inverted repeats IR-2 L and IR-2 R at nucleotide 1097 in Fig. 2; however, since T's rarely represent start sites for transcription, a more likely candidate was the adjacent (downstream) A. The differences in start sites may represent a "stuttering" of the initiation event (5). Alternatively, it may be that initiation starts at one of the upstream G's and that the differences reflect degradation of the 5′ end of the message.

To confirm that *gtfG* possesses its own promoter and that its transcription is regulated by *rgg*, we examined its regulation in *E. coli*. We constructed an *E. coli gtfG* transcriptional reporter strain designated VA204, a derivative of *E. coli* MC4100 [F$^-$ *araD139* (Δ*lac*)*U169 rpsL relA thi*] that is lysogenic for a recombinant lambda phage containing a *gtfG′-lacZ* transcriptional fusion. The fusion was constructed as follows. A DNA fragment extending from the *Sal*I site located upstream of the *rgg* stop codon to the *Nsi*I site located 32 bp downstream from the *gtfG* start codon was isolated from pAMS29b (21). *Bam*HI linkers were added, and the fragment was digested with *Eco*RI and *Bam*HI to yield a 135-bp fragment that contained the sequence between *rgg* and *gtfG* flanked by 36 bp at the 3′ end of *rgg* and 32 bp at the 5′ end of *gtfG*. It was ligated to the *Eco*RI- and *Bam*HI-digested transcriptional reporter vector pRS551 (18) that encodes a kanamycin resistance determinant and was introduced into MC4100; by this procedure, the *gtfG*-related DNA was directed in the same orientation as that of *lacZ*. This strain was infected with λRS88 (*imm434 ind*$^-$) (18) to create a lysate containing recombinant phage subsequently used to infect MC4100. Selection on kanamycin yielded strain VA204, a lysogen with the *gtfG′-lacZ* fusion located at the lambda attachment site. Another lysogen produced from a lambda lysate that was generated by infection of MC4100 (pRS551) was also constructed as a control and designated VA203. Strain VA204 had β-galactosidase levels 100-fold higher than those of strain VA203 (Table 1). Thus, the *gtfG′-lacZ* fusion was active in *E. coli*, suggesting that there was *gtfG* promoter activity *E. coli*.

To evaluate whether the *gtfG* promoter could be regulated, *rgg* was cloned onto a multicopy plasmid pBC SK$^+$ (Stratagene, La Jolla, Calif.) and introduced into the reporter fusion and control *E. coli* strains. The construction of the vector containing *rgg* was as follows. A single 1,499-bp fragment from *Nsi*I-digested pAMS29b was ligated to the *Pst*I site in pBC SK$^+$, yielding pRGG. This insert contained the entire *rgg* flanked by 511 bp of DNA upstream of the *rgg* start codon and by 32 bp of the 5′ end of *gtfG* and was cloned into the multiple-cloning site in an orientation opposite to that of *lacZ* transcription to ensure that the vector sequences did not influence expression of *rgg*. When pRGG was introduced into VA204 there was a 20-fold increase in *gtfG′-lacZ* fusion expression compared with that of the same strain containing the control plasmid pBC SK$^+$ (Table 1). The data show that *gtfG* had its own promoter activity, which was confined to a region between *rgg* and *gtfG*, and that it was transcriptionally regulated by *rgg* in *E. coli*. The

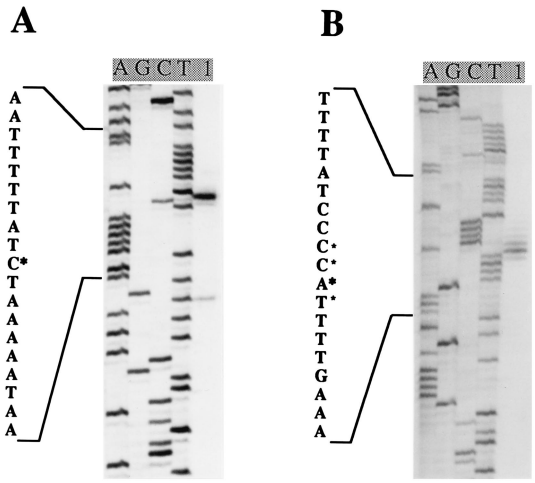

FIG. 3. Identification of *rgg* (A) and *gtfG* (B) transcriptional start sites by primer extension. Primer extension was done with reverse transcriptase, total RNA from *S. gordonii* CH1 containing plasmid pAM-S40, and synthetic primers located near the 5′ ends of both genes (see oligonucleotides A and B in Fig. 2). The primers were end labeled with [γ-^{32}P]ATP and polynucleotide kinase (GIBCO BRL) by the procedure of Sambrook et al. (13). Primer extension was carried out by the method of Ausubel et al. (1) with total RNA from CH1 containing pAM-S40, avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.), and the above-mentioned primers as the templates. Double-stranded DNA sequencing was accomplished by a modification (19) of the dideoxy chain termination sequencing method (14) with a Sequenase sequencing kit (United States Biochemicals, Cleveland, Ohio), plasmid pAMS-29b as the template, a plasmid which contains *rgg* and the 5′ end of *gtfG* (21), and the above-mentioned primers. Both primer extension reaction products and DNA sequencing reaction products were subjected to gel electrophoresis and autoradiographed as described by Ausubel et al. (1). The sequences to the left of the autoradiograms are sequences determined from the sequencing lanes A, G, C, and T. The nucleotide residues that comigrate with the primer extension product in lanes 1 are marked by asterisks.

data also suggest that *rgg* was expressed in *E. coli* from its own promoter.

To conclude, transcripts that correspond to the *S. gordonii rgg* and *gtfG* loci were identified, and the locations of the 5′ ends of the transcripts were determined. The results of Northern blot analysis imply that the *rgg* gene product is a positive regulator of transcriptional expression of *gtfG*, since a frameshift mutation in *rgg* resulted in the absence of a 5.4-kb *gtfG*-specific transcript; complementation of the lesion in *trans* by a multicopy vector carrying *rgg* restored the transcript. The RNA study was corroborated by transcriptional reporter fusion studies, although the confirmation was done in a heterologous background.

One interpretation of our results is that titration of a negative regulator by DNA sequences flanking *rgg* on the multicopy plasmid leads to enhanced *gtfG* expression. This is unlikely since we have previously shown that GTF activity is not increased in a strain containing the same plasmid with a frameshift mutation in *rgg* (21) and also since we have shown regulation in *E. coli*, a host that is likely not to have a *gtfG* promoter-specific negative regulator. The relevance of this work with respect to the previously reported Spp$^+$-Spp$^-$ phase variation phenomenon (22) is being investigated. It would be interesting to determine if there are specific physiological conditions similar to those described by other investigators for various streptococcal species (2, 3, 4, 7, 8, 15, 23, 27) that affect expression of the *S. gordonii* Challis GTF enzyme and, if so, to determine the possible involvement of *rgg*.

TABLE 1. Regulation of the *gtfG′-lacZ* operon fusion by *rgg* in *E. coli*

Strain	Fusion genotype[a]	Plasmid	β-Galactosidase activity[b]
VA203	Tl_4-*lacZ*	pBC SK$^+$	1
VA203	Tl_4-*lacZ*	pRGG	2
VA204	Tl_4-*gtfG′-lacZ*	pBC SK$^+$	89
VA204	Tl_4-*gtfG′-lacZ*	pRGG	2,001

[a] Tl_4 is four tandem copies of the terminator of the *E. coli rrnB* operon.
[b] β-Galactosidase activity was measured by the method of Miller (10) from cells harvested at mid-log phase growth in Luria-Bertani broth (Difco). Activity is expressed in Miller units.

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