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Identification of a Gene, *rgg*, Which Regulates Expression of Glucosyltransferase and Influences the Spp Phenotype of *Streptococcus gordonii* Challis

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***Streptococcus gordonii* Challis was previously shown to give rise to phase variants expressing high (Spp$^+$) or low (Spp$^-$) levels of extracellular glucosyltransferase (GTF) activity. Here, shotgun cloning of an *S. gordonii* Spp$^+$ chromosomal digest resulted in a chimeric plasmid (pAM5010) able to complement the Spp$^-$ phenotype. In addition, introduction of pAM5010 into an Spp$^+$ strain resulted in a 10-fold increase in GTF expression. Deletion analysis of pAM5010 identified a 1.2-kb DNA segment which exhibited the same functional properties as pAM5010. Nucleotide sequence analysis of this region revealed a gene approximately 1 kb in size. The gene was designated *rgg*. Disruption of the chromosomal *rgg* gene open reading frame in an Spp$^+$ strain resulted in strain DS512, which displayed an Spp$^-$-like phenotype and had 3% of wild-type GTF activity. A plasmid containing the *rgg* gene was able to complement the DS512 phenotype and significantly increase GTF expression above wild-type levels. Sequence analysis and other data showed that the *S. gordonii* GTF determinant, designated *gtfG*, is located 66 bp downstream of the *rgg* gene. The sequence also revealed interesting inverted repeats which may play a role in the regulation of *gtfG*. We conclude that *rgg* positively regulates the expression of GTF and influences expression of the Spp phenotype.**

Streptococcus gordonii was, until recently, classified as *Streptococcus sanguis* (25). It is considered a noncariogenic (19, 20, 36) inhabitant of the supragingival mature plaque and is also found in the oropharyngeal mucosa (12). Along with *S. sanguis*, it is commonly associated with infective endocarditis (3, 38). It is believed that, as the result of dental procedures or poor oral hygiene, the organisms can enter the bloodstream, resulting in bacteremias which could progress to endocarditis (4).

The ability of *S. gordonii* to produce extracellular polysaccharide may be important in the accumulation of bacteria on smooth tooth surfaces and perhaps also in the organism's ability to cause endocarditis. *S. gordonii* shares with the cariogenic mutans streptococci the ability to produce the extracellular enzyme glucosyltransferase (GTF; EC 2.4.1.5). GTF hydrolyzes sucrose and uses the glucosyl moiety to synthesize water-soluble or -insoluble polysaccharides (glucan) (32). It is well established that insoluble glucan produced by the mutans streptococci is important for bacterial accumulation on smooth tooth surfaces and that such accumulation can lead to smooth-surface dental caries (13, 18). Although the role of glucan synthesis by *S. sanguis* and *S. gordonii* has not been determined, recent in vitro evidence showed that glucan production can lead to the accumulation of *S. gordonii* on hydroxyapatite surfaces (55). As for the role of GTF in infective endocarditis, in vivo and in vitro studies by Ramirez-Ronda (39) and Scheld et al. (45) suggested that *S. gordonii* and *S. sanguis* glucan production may contribute to virulence.

Genetic regulation may be responsible for the changes in levels of GTF observed when bacteria are grown under different conditions. *Streptococcus mutans* Ingbritt (58) and OMZ176 (57) displayed increased GTF levels when grown at elevated growth rates. The nonionic detergent Tween 80 acts to decrease extracellular GTF activity in *S. gordonii*, presumably by its action on membrane fluidity (6, 24). Increases in sodium ion concentrations decrease extracellular GTF activity in *S. gordonii* (6). Thus, growth rate, neutral detergents, and sodium ion concentration may affect GTF expression. A molecular genetic analysis of regulation of GTF expression was recently conducted in *S. mutans* GS-5, with the result that sucrose was able to induce transient increased expression of GTF (23). Recently, two mutants of *S. gordonii* Challis were shown to express increased levels of GTF expression (17); this suggests that GTF regulatory genes may exist.

Recently, we reported a phase variation phenomenon whereby *S. gordonii* Challis reversibly switched from a hard-colony phenotype (Spp$^+$) on medium containing sucrose to a soft-colony phenotype (Spp$^-$) (53). The Spp$^-$ colonies were able to switch back to the Spp$^+$ phenotype. The phase variation occurred at a frequency of 10^{-3} to 10^{-4} and was correlated with differences in levels of detectable GTF. There was generally a 4- to 10-fold difference in GTF activity, with Spp$^+$ cells having the higher levels. In this article, a gene is described which functionally complements Spp$^-$ phase variants and also positively regulates *gtf* expression. The gene maps just upstream of the *gtf* determinant.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this study, together with their characteristics, related references, and sources, are listed in Table 1. *S. gordonii* CH1 is a Challis strain which exhibits a characteristic hard-colony phenotype on Todd-Hewitt broth (THB) containing 3% sucrose. This

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Bacteria		
S. gordonii CH1	Strain Challis; Spp^+	Strain V288; 31
S. gordonii CH97	CH1 phase variant; Spp^-	53
S. gordonii DS511	CH1 containing chromosomally integrated pAM-S51; Em^r	This study
S. gordonii DS513	CH1 (*rgg*$^+$); Em^s Spp^+	This study
S. gordonii DS512	CH1 (*rgg*); Em^s Spp^-	This study
E. coli P2392	LE392 (P2 lysogen)	Stratagene
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44*λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Labs
Phages		
λ Dash II	λ cloning vector; *red*$^+$ *gam*$^+$	Stratagene
λS6	λ Dash II with CH1 genome fragment; GTF^+ in DH5α	This study
Plasmids		
pVA891	*E. coli* vector; Em^r	30
pVA749	Streptococcal cloning vector; Em^r	31
pBluescript SK	*E. coli* vector; Ap^r	Stratagene
pBluescript KS	*E. coli* vector; Ap^r	Stratagene
pAM5010	pVA749 with CH1 chromosomal fragment insert	This study
pAM-S13	Subclone of pAM5010	This study
pAM-S42	Subclone of pAM5010	This study
pAM-S43	Subclone of pAM5010	This study
pAM-S44	Subclone of pAM5010	This study
pAM-S45	Subclone of pAM5010	This study
pAM-S57	Subclone of pAM5010	This study
pAM-S51	pVA891 with pAM-S44 insert fragment	This study
pAM-S29b	pBluescript SK containing pAM-S13 *Hin*dIII insert fragment	This study
pAM-S36	pBluescript KS containing pAM-S13 *Hin*dIII insert fragment	This study

phenotype was previously designated Spp^+ (sucrose-promoted phenotype positive [53]). CH1 gives rise to phase variants (Spp^-), such as CH97, which are soft on the same media (53); the phenomenon is reversible.

Media. *S. gordonii* strains were grown at 37°C in THB (Difco Laboratories, Detroit, Mich.). To distinguish their colonial Spp phenotype, they were grown at 37°C in a candle jar or anaerobically (Gas Pak Plus Anaerobic Jar System; BBL, Cockeysville, Md.) for 2 to 3 days on THB solid medium containing 3% sucrose. *Escherichia coli* DH5α was grown at 37°C in LB medium (2). *E. coli* DH5α containing recombinant pBluescript plasmids was identified as white colonies on LB solid medium containing 0.5 mM isopropyl-β-D-thiogalactopyranoside and 12 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (both from Sigma). *E. coli* P2392 was grown at 37°C in NZCYM medium (43). When present in selective plates, antibiotics (Sigma, St. Louis, Mo.) were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and erythromycin, 5 μg/ml.

Preparation of DNA. Sarkosyl lysates of *S. gordonii* and *E. coli* were prepared essentially as described by Clewell et al. (7). *S. gordonii* was grown overnight in THB containing 0.5% glycine; following centrifugation, the cell pellet was incubated in lysozyme (1 mg/ml) and 50 U of mutanolysin (Sigma). After cell lysis, two isopycnic CsCl-ethidium bromide gradient centrifugations were done. For preparation of small amounts of *E. coli* plasmid DNA, the alkaline-detergent method was used (2). A similar procedure was used to obtain small amounts of *S. gordonii* plasmid DNA (9), with the following modifications: 5 ml of cells was grown overnight in 0.5% glycine and, after being harvested, incubated in a lysozyme solution which included 50 U of mutanolysin. Bacteriophage DNA from *E. coli* was prepared by the polyethylene glycol standard method as described by Sambrook et al. (43).

DNA manipulations. General procedures for cloning and DNA manipulations were essentially as described by Sambrook et al. (43). Restriction endonuclease digestions and ligations with T4 DNA ligase were done as recommended by the suppliers (GIBCO BRL, Grand Island, N.Y., and Boehringer Mannheim, Indianapolis, Ind.). DNA fragments to be used for cloning and hybridization probes were isolated from agarose by the QIAEX gel extraction protocol (Qiagen Inc., Chatsworth, Calif.). The Nick Translation System (GIBCO BRL) was used according to the supplier's protocol to label DNA probes with [α-^{32}P]dATP (Amersham, Arlington Heights, Ill.). Plasmid deletion derivatives (see Fig. 2) were constructed by digestion of pAM5010 with appropriate restriction endonucleases which flank the DNA segment to be deleted and religation of the plasmid. In some cases, mung bean nuclease (GIBCO BRL) was used to produce blunt-ended DNA prior to ligation. The plasmid pAM-S13 was derived from pAM5010 and was used to generate all other deletion derivatives.

Transformation of bacteria. The genetic transformation protocol of Lawson and Gooder (28) was used to introduce plasmids into *S. gordonii* except that horse serum (Colorado Serum Co., Denver, Colo.) was used instead of swine serum. *E. coli* was transformed by the standard $CaCl_2$ method (43).

Lambda library construction and plaque hybridization. A partial *Sau*3A restriction digest of *S. gordonii* Challis chromosomal DNA was ligated to *Bam*HI-digested Lambda Dash II. After in vitro packaging with Gigapack (Stratagene, La Jolla, Calif.), the library was used to infect *E. coli* P2392. Plaques were screened by hybridization as described by Ausubel et al. (2) with the 1.5-kb *Hin*dIII insert fragment of pAM5010 as a probe. Six plaques hybridized to the probe.

One selected plaque contained the lambda recombinant λS6, which exhibited GTF activity in *E. coli* P2392 lysates (data not shown). λS6 also contained the same 4-kb *Hin*dIII insert fragment found in pAM5010.

Nucleotide sequence determination. The 4-kb *Hin*dIII fragment from λS6 was cloned into the *Hin*dIII site of pBluescript KS and pBluescript SK, and the plasmids were designated pAM-S29b and pAM-S36, respectively. The exonuclease III-mung bean nuclease procedure described by Stratagene was used to create nested deletions of pAM-S29b and pAM-S36 in both orientations. Double-stranded DNA sequencing was done by a modification (51) of the dideoxy chain termination sequencing method (44) with the Sequenase sequencing kit, universal sequencing primers, and M13 reverse primers (United States Biochemical Corp., Cleveland, Ohio). Unique synthetic primers were also made (DNA Core Facility, University of Michigan, Ann Arbor, Mich.) to complete the sequence on both DNA strands.

Individual sequences were stored and matched by using the DNA Inspector IIe software program (Texto, Lebanon, N.H.). Sequence analysis was performed with the IBI Mac Vector software program (GenBank Database Release 67.0, March 1991 [International Biotechnologies, Inc., New Haven, Conn.]). The free energy (ΔG) of RNA secondary structure was calculated by Salser's energy model (42).

Assay of GTF activity. Cell growth conditions and the method used for measuring GTF activity were as described by Tardif et al. (53), based on previously described protocols (41, 59). Briefly, supernatant samples were obtained from late-log-phase batch cultures. All cultures were grown to 150 Klett units (measured with a Klett-Summerson colorimeter [no. 54 filter]); cell dry weights were estimated by weighing cell pellets which had been washed in distilled water and dried at 105°C for 24 h, and the protein concentrations of the supernatants (A_{280} measured spectrophotometrically) were determined. No significant differences in dry weights or protein concentrations were observed (Student's paired *t* test) between the wild-type CH1 strain and the other strains assayed. Samples were subjected to sodium dodecyl sulfate–8.75% polyacrylamide gel electrophoresis. The gel was incubated in a solution of Triton X-100 and sucrose. A glucan polymer band forms where the *S. gordonii* CH1 GTF enzyme band is located on the gel at 170 kDa (53). The glucan was stained by the periodic acid-Schiff method (59). Laser densitometry (LKB Ultroscan XL) provided a relative quantitative value of GTF activity by comparison of band intensities with that of wild-type CH1 (100%). Relative band intensities were shown by Tardif et al. (53) to correspond to the relative amounts of GTF activity as determined by the [^{14}C]glucose incorporation procedure (26). When culture suspensions of CH1 (Spp^+) or CH97 (Spp^-) cells were used as the starting material, extracts assayed for GTF by the activity gel method exhibited levels that were essentially the same as those of culture supernatants; thus, significant amounts of cell-associated GTF were not evident.

Southern hybridization. DNA was cut with restriction endonucleases and run on a 0.7% agarose gel, transferred to nitrocellulose (Optibind; Schleicher & Schuell, Keene, N.H.), and hybridized under stringent conditions to nick-translated radioactive DNA fragments as described by Ausubel et al. (2), based on the method of Southern (48).

Nucleotide sequence accession number. The GenBank sequence accession number for the nucleotide sequence shown in Fig. 3 is M89776.

RESULTS

Cloning of a DNA fragment which complements the Spp^- phenotype. On solid medium containing sucrose, *S. gordonii* CH1 colonies are hard and cohesive and adhere to the agar. This represents the sucrose-promoted phenotype (Spp^+) and is shown in Fig. 1A. Figure 1B shows an Spp^- phase variant with its soft, noncohesive, and nonadherent phenotype. A partial *Hin*dIII restriction digest of *S. gordonii* CH1 chromosomal DNA was ligated to the *Hin*dIII-digested streptococcal vector pVA749 and introduced into the Spp^- *S. gordonii* strain CH97 by transformation. An erythromycin-resistant (Em^r) Spp^+ transformant was obtained which contained the vector ligated to a 5.5-kb partial *Hin*dIII fragment. This plasmid was denoted pAM5010 (Fig. 2). A 1.5-kb *Hin*dIII fragment was deleted from pAM5010, resulting in pAM-S13 (Fig. 2). Deletion of this chromosomal *Hin*dIII fragment did not affect the Spp^+ phenotype of the clone. This delineated a region of 4 kb which could complement the Spp^- phenotype of the phase variant CH97. Complementation is seen in the photograph of the Spp^+ CH97(pAM-S13) colony phenotype in Fig. 1C. The CH97(pAM-S13) phenotype is similar to the wild-type CH1 colony phenotype in Fig. 1A and contrasts sharply with the CH97 phenotype shown in Fig. 1B. pAM5010 and pAM-S13 were also able to complement two other Spp^- variants, CH1A8 and CH1C1 (data not shown).

Localization of the DNA region which complements the Spp^- phenotype. Deletion derivatives were constructed from pAM5010 as described in Materials and Methods and are illustrated in Fig. 2. A 4-bp deletion present in pAM-S44 resulted from elimination of the *Sal*I site (verified by DNA sequencing). All plasmids were transformed into the Spp^- strain CH97 to determine whether they would restore the Spp^+ phenotype. Deletion derivatives retaining the ability to complement the Spp^- phenotype identified the portions of DNA that were not necessary for complementation. The region between the vertical dotted lines in Fig. 2 delineates the segment of DNA sufficient for complementation of the Spp^- phenotype. The 1.2-kb DNA region within the dashed lines was termed RSC, for region of Spp complementation.

Effect of localized DNA region on Spp phenotype and GTF activity. The plasmid derivatives which complemented the Spp^- phenotype of CH97 (pAM5010, pAM-S13, pAM-S45, and pAM-S57), when present in wild-type strain CH1, appeared to increase the intensity of the sucrose-promoted phenotype. This phenotype was designated Spp^{+++} and is shown in Fig. 1E. It is characterized by a colony type similar to the wild type (Fig. 1A) but is more crusty-looking on medium containing sucrose. It is also more adherent and cohesive than the wild type on the same medium. Deletion derivatives which did not complement the Spp^- phenotype also conferred no phenotypic change on wild-type CH1.

The Spp^{+++} CH1 transformants which contained plasmids carrying RSC had approximately 10-fold more extracellular GTF activity than the wild-type control CH1 (pVA749) (see GTF activity for pAM5010, pAM-S13, pAM-S45, and pAM-S57 in Fig. 2). All the derivatives which did not restore the Spp phenotype in CH97 also did not increase extracellular GTF activity levels above the wild-type level (see GTF activity column for pAM-S42, pAM-S43, and pAM-S44 in Fig. 2). Southern blot analysis of the CH1 transformant containing pAM-S13 showed no evidence of plasmid integration (data not shown). In addition, Spp^+ and Spp^- phase variants containing pAM5010 or pAM-S13 displayed elevated extracellular GTF activity (data not shown).

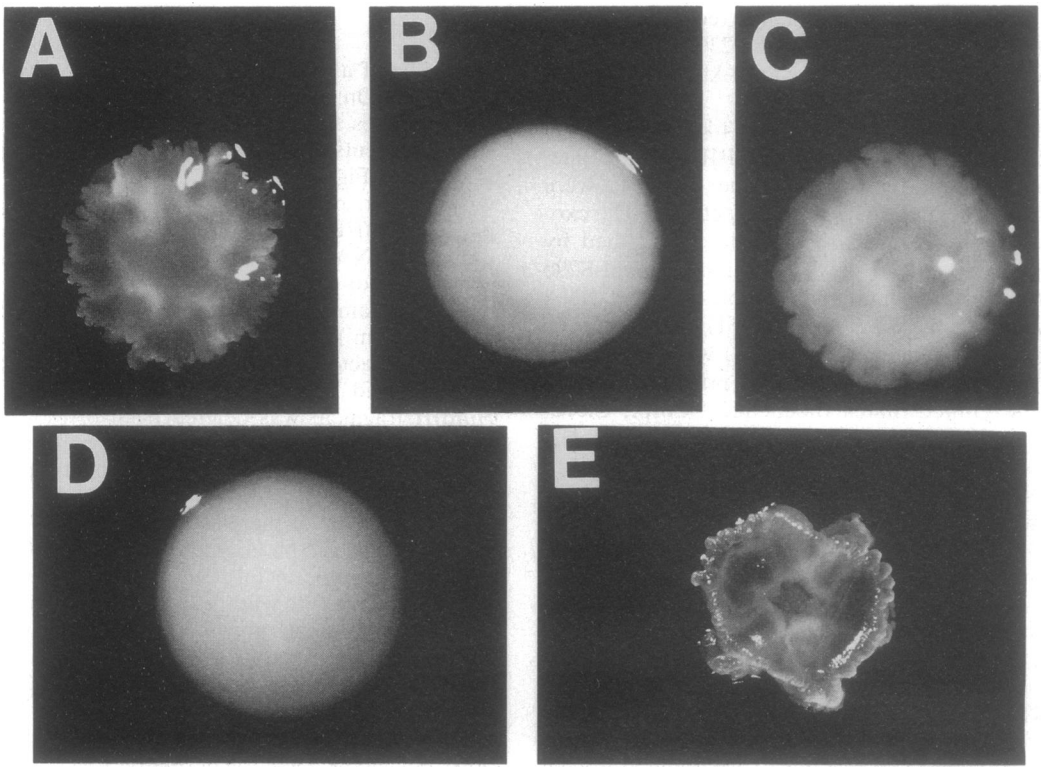

FIG. 1. Photographs of colonies of different *S. gordonii* strains grown for 3 days in candle jars at 37°C on THB medium containing 3% sucrose. (A) Spp^+ *S. gordonii* CH1; (B) Spp^- phase variant CH97; (C) CH97(pAM-S13); (D) DS512, *rgg* mutant of CH1; (E) DS512(pAM-S57).

Therefore, the RSC segment on the vector acts in *trans* to (i) complement the Spp^- phase variants and (ii) change the wild-type phenotype to Spp^{+++}. We have named this locus *rgg*, for regulator gene of GTF.

Cloning of the 4-kb *Hin*dIII fragment containing *rgg* for sequencing. As noted above, the 4-kb *Hin*dIII fragment conferring the Spp^+ phenotype was cloned directly in the variant CH97. When considering use of this DNA for nucleotide-sequencing purposes, we were concerned about the possibility that an allelic exchange between the insert in pAM-S13 and the chromosomal allele of CH97 may have occurred. If this were true, the "mutated" chromosomal allele might now be contained within pAM-S13. Therefore, another 4-kb CH1 chromosomal *Hin*dIII fragment which hybridized to the pAM-S13 insert was cloned from an *S. gordonii* Challis partial *Sau*3A Lambda Dash II library and subsequently subcloned into pBluescript SK and pBluescript KS to yield two recombinant plasmids, pAM-S29b and pAM-S36, respectively. This provided us with a "wild-type" allele for subsequent sequencing analysis.

Sequence of the *rgg* locus. The sequence shown in Fig. 3 contains the RSC. As evident from Fig. 3, there is only one complete open reading frame, denoted ORF-1; we believe that this represents the *rgg* gene (see below). ORF-1, encoding 297 amino acid residues, begins with the ATG codon at nucleotide 173 and terminates with a TAG stop codon at nucleotide 1066. Located 6 bp upstream of the ORF is a possible Shine-Dalgarno ribosome-binding site (10, 34, 46). A potential promoter is located 50 bp upstream of the start codon, with a −35 (TTGCCA) and a −10 (TAAAAT) consensuslike (40) sequence. An interesting feature is a region of dyad symmetry (IR-1) situated between the −10 promoter site and the ribosome-binding site (see Discussion). Although this region is AT rich, similar regions of dyad symmetry of this size (26 nucleotides) could not be found in the remainder of the sequence shown in Fig. 3. Following ORF-1 is a large inverted repeat (IR-2) which could give rise to two different stable RNA structures (see Fig. 6). The first structure (see Fig. 6A) is a possible RNA factor-independent transcription termination hairpin (5), consisting of a 17-bp stem and a 20-bp loop. The second (see Fig. 6B) can also form a stable RNA hairpin with a larger stem of 22 bp. This second structure lacks the run of U's following the stem loop typically seen in factor-independent terminators (see Discussion). The putative ribosome-binding site of a downstream open reading frame, ORF-2, is located within the stems of the putative RNA secondary structures.

ORF-1 encodes a protein with a calculated molecular weight of 34,436 and an isoelectric point of 5.8. No signal sequence (37) or membrane-spanning regions (27) were detected in the amino acid sequence, suggesting that ORF-1 encodes a cytoplasmic protein. ORF-1 had no significant overall homology with any protein in the GenBank data base.

ORF-2 lies 63 bp downstream of the ORF-1 stop codon. It begins at nucleotide 1130 with an ATG start codon and does not terminate within the region shown in Fig. 3 (96 amino acids are shown). The ORF is preceded by a consensus ribosome-binding site (GGAGG) and putative promoter sequences 38 bp upstream of the start codon, with a −10 (TAAAAT) and a −35 (TAGAAC). The inverted repeat IR-2 (see above) encompasses the ribosome-binding site and promoter. ORF-2 has a typical signal sequence in the N-terminal region (37) (underlined in Fig. 3) and a consensus

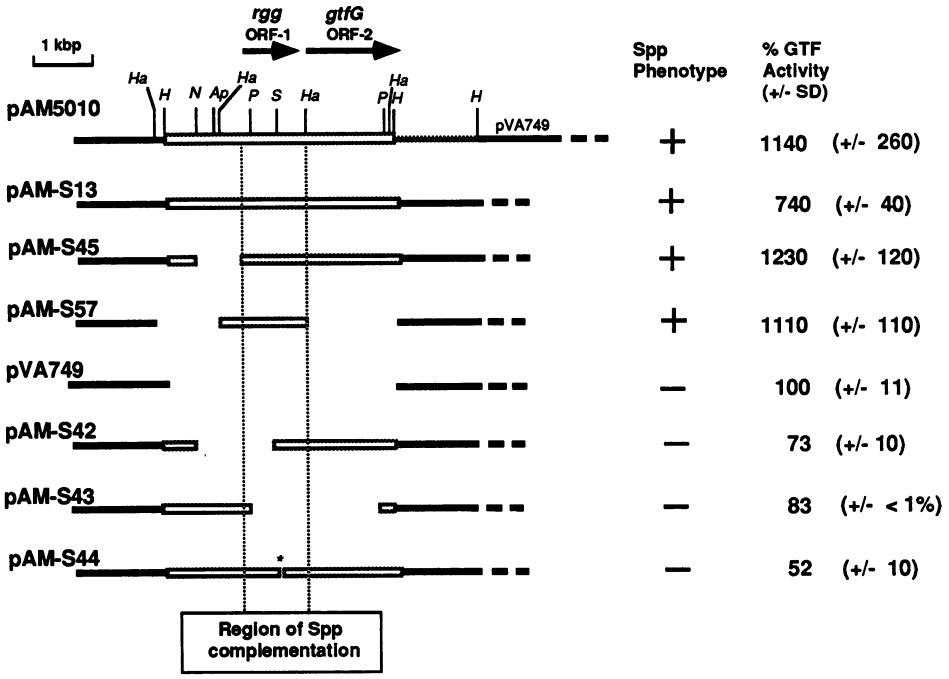

FIG. 2. Schematic of plasmid derivatives, depicting the region which complements the Spp$^-$ phase variants and relative levels of extracellular GTF. The restriction map of pAM5010 is shown. Ha, *Hae*III site; H, *Hin*dIII site; N, *Nru*I site; Ap, *Apa*I site; P, *Pvu*II site; S, *Sal*I site. A + in the Spp phenotype column indicates that a plasmid derivative confers an Spp$^+$ phenotype on the Spp$^-$ phase variant CH97. This was determined by inspection of CH97 transformed with the various derivatives. A − indicates that no change in the Spp$^-$ CH97 phenotype occurred. The GTF activity of plasmid derivatives placed in *S. gordonii* CH1 was determined relative to that from CH1 containing the control plasmid pVA749 (100%). GTF from supernatants of cultures of CH1 transformed with plasmid derivatives was assayed and quantitated as described in Materials and Methods. All samples were assayed in triplicate, and a standard deviation (SD) was calculated. The asterisk in the pAM-S44 schematic denotes a 4-bp deletion which resulted in the absence of the *Sal*I restriction site.

cleavage site (56) after amino acid 35, suggestive of an exported protein. Significant homology (33 to 38% conserved amino acid similarity) was seen with ORF-2 and the N-terminal deduced amino acid sequences of *S. sobrinus* 6715 *gtfI* (1), *S. downei* MFe28 *gtfI* (11), *S. mutans* GS-5 *gtfD* (22) and *gtfC* (54), and *S. downei* MFe28 *gtfS* (14). This similarity suggests that ORF-2 is a *gtf* gene, and other evidence strongly supports this. First, continuation of the DNA sequence downstream of that presented here (52; data not shown) revealed that ORF-2 encoded at least 557 amino acids, with a 345-amino-acid region which displayed greater than 50% identity and greater than 60% conserved amino acid sequence similarity to most of the streptococcal *gtf* genes sequenced to date; that is, 65% amino acid identity was seen with an equivalent region of *gtfD* (22), 56% with *gtfC* (54), 54% with *gtfI* (11) and *gtfB* (47), and 51% with *gtfS* (14). Second, no translational stop site was encountered in the 1,671 nucleotides of ORF-2, which means that ORF-2 encodes a protein larger than 61 kDa; this is consistent with evidence that *S. gordonii* GTF is 170 kDa (53). Third, a lambda clone containing ORF-1, ORF-2, and downstream DNA expressed GTF activity from *E. coli* (data not shown; see λS6 in Table 1). Fourth, insertion mutagenesis in the Challis chromosome 1,700 bp downstream of the region of ORF-2 shown in Fig. 3 resulted in complete absence of GTF activity (data not shown). An abundance of data therefore indicate that ORF-2 is the *S. gordonii gtf* gene, and we have designated it *gtfG*. When DNA carrying a significant portion of the *gtfG* gene was used as a hybridization probe in Southern blot hybridization experiments, only one determinant (i.e., *gtfG*) could be detected. Thus, there do not appear to be closely related *gtf* determinants elsewhere on the *S. gordonii* chromosome.

Mutagenesis of the CH1 chromosomal *rgg* locus results in an Spp$^-$ phenotype with significantly reduced GTF activity. The plasmid pAM-S44 (Fig. 2) has a 4-bp deletion which results in a frameshift mutation in *rgg*. The mutation predicts termination 10 amino acids downstream of the lesion (Fig. 3; see asterisk below DS512 at nucleotide 786). The *Hin*dIII fragment of pAM-S44, containing the *rgg* mutation, was ligated to the unique *Hin*dIII site of pVA891 (a plasmid which is unable to replicate in *S. gordonii* and contains a determinant [*erm*] encoding resistance to erythromycin, a selectable marker in *S. gordonii*). This new plasmid was designated pAM-S51. It was used to transform CH1 to Em^r, resulting from its integration into the CH1 chromosome via a reciprocal recombination. One transformant obtained was chosen for further study and was designated DS511. Southern blots of DS511 chromosomal DNA digested with *Hin*dIII and *Sal*I were transferred to nitrocellulose and hybridized to the pAM-S13 4-kb *Hin*dIII fragment probe. The data reveal (Fig. 4, lane 2) a 4-kb band representing the mutant allele (*Sal*I will not digest at the mutation site) and two ca. 2-kb bands (*Sal*I digests the wild-type *Hin*dIII fragment into two equal-sized fragments). Note that CH1 DNA digested with the same enzymes yields two ca. 2-kb bands (Fig. 4, lane 1), indicative of the wild-type allele. Growth of the resultant Em^r colonies under nonselective conditions gave rise to an erythromycin-sensitive (Em^s) Spp$^-$ strain, designated DS512, and an Em^s Spp$^+$ strain, designated DS513. These strains resulted from the loss of the integrated pVA891 chimera mediated by recombination of the flanking homolo-

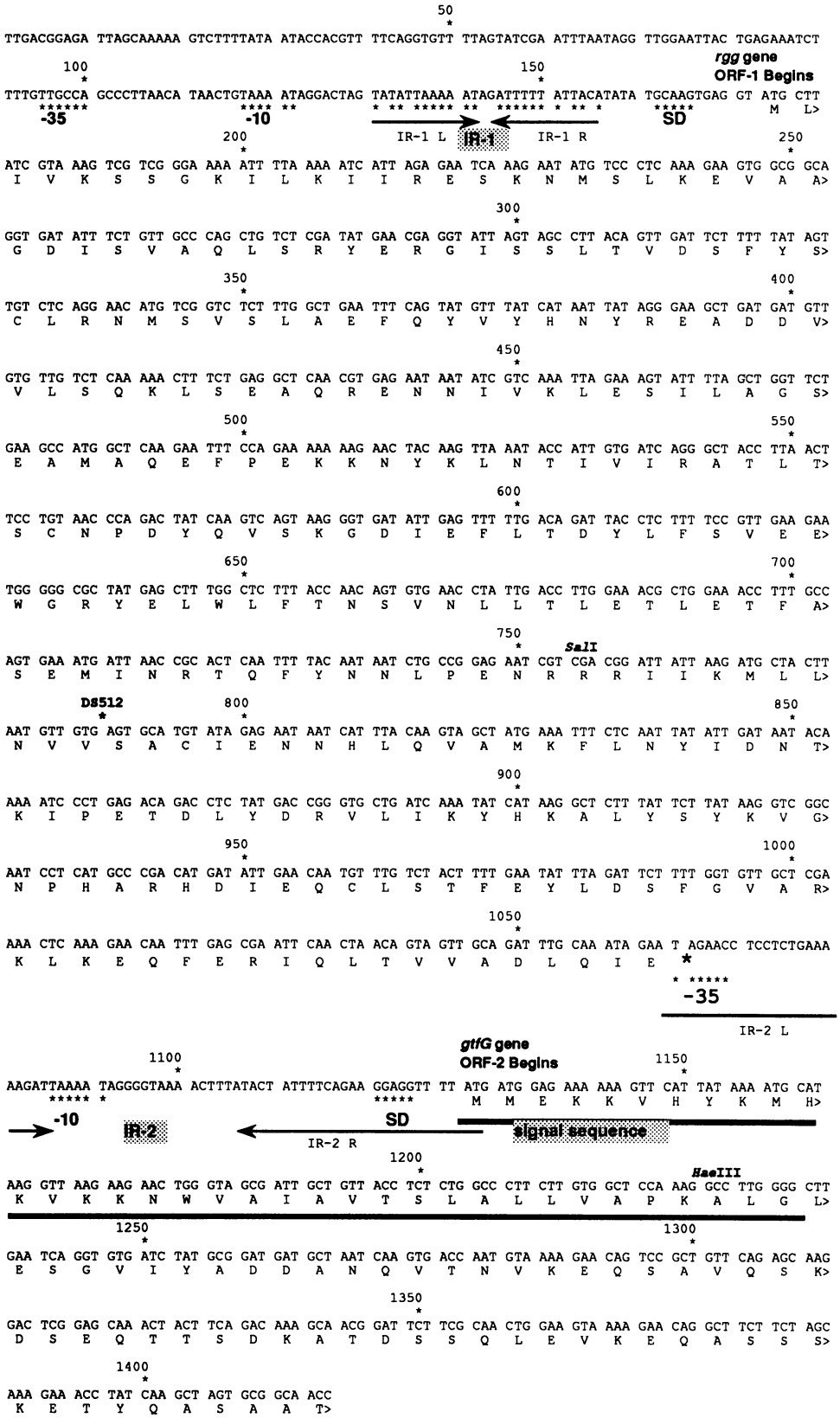

FIG. 3. Nucleotide sequence of the *rgg* gene and flanking sequences, which include the 5′ end of the *gtfG* gene. The sequence is the region of Spp complementation shown in Fig. 2 with an additional 189 bp at the 3′ end. The deduced amino acid sequences are shown below the nucleotide sequence, and the asterisk at the end of the *rgg* open reading frame denotes a stop codon. Putative promoters are indicated by asterisks at the −10 and −35 sites, and putative ribosome-binding sites are denoted by asterisks above SD. Inverted repeats IR-1 (composed of IR-1L and IR-1R) and IR-2 (composed of IR-2L and IR-2R) are underlined. The asterisk labeled DS512 denotes where translation of *rgg* terminates when a 4-bp deletion resulting in a frameshift mutation is created at the *Sal*I site at nucleotide 753. The signal sequence of the *gtfG* open reading frame, denoted by a heavy line, ends at the glycine (G) residue.

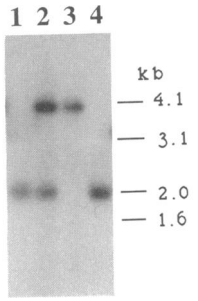

FIG. 4. Southern blot analysis showing the absence of a *Sal*I digestion site in the chromosomal DNA of *S. gordonii* DS512. Chromosomal DNA was double-digested with *Hin*dIII and *Sal*I, run on a 0.7% agarose gel, transferred to nitrocellulose, probed with the 4-kb insert fragment of pAM-S13, and autoradiographed as described in Materials and Methods. The DNA size markers are shown to the right of the figure. Digested DNA was from *S. gordonii* CH1 (lane 1), DS511 (lane 2), DS512 (lane 3), and DS513 (lane 4).

gous DNA. In this process, either the wild-type or the mutant *rgg* allele of pAM-S51 can be lost. The DS512 Spp^- chromosomal DNA, when digested and probed as described above, had the mutant allele (Fig. 4, lane 3), while the DS513 Spp^+ chromosomal DNA displayed a wild-type hybridization pattern (Fig. 4, lane 4). Washing and probing the same blot with pVA891 revealed that pVA891 was present in the original transformant DS511 but absent in both DS512 and DS513 (52; data not shown). As expected, the DS512 (Spp^-) strain did not appear to undergo phase variation to the Spp^+ phenotype (i.e., $<4 \times 10^{-5}$ frequency of switching).

A colony of the *rgg* mutant DS512 is shown in Fig. 1D and contrasts markedly with the Spp^+ wild-type CH1 colony in Fig. 1A. A significant decrease in extracellular GTF activity (Fig. 5, lane 2) relative to the GTF activity of wild-type CH1 (lane 1) was found. Laser densitometry tracings indicated that DS512 produced only 3% of the wild-type level of GTF.

Complementation of Challis *rgg* mutant strain with a plasmid containing *rgg* results in restoration of the Spp^+ phenotype and increased GTF levels. The plasmid pAM-S57 (Fig. 2) was transformed into the mutant strain DS512. The resulting Em^r transformants were Spp^{+++}, with about sixfold more GTF activity than the wild type, as assessed by GTF activity gel analysis. The band intensity of DS512(pAM-S57) (Fig. 5, lane 3) is dramatically increased above that of DS512(pVA749) and CH1(pVA749) (Fig. 5, lanes 2 and 1, respectively). The Spp^+ colonial phenotype of DS512(pAM-S57), shown in Fig. 1E, clearly contrasts with that of the Spp^- DS512 in Fig. 1D. Therefore, the mutation in *rgg* which decreases GTF levels and produces an Spp^- phenotype in CH1 can be complemented by the *rgg* gene supplied in *trans*. Indeed, the increased copy number present when *rgg* was on the plasmid appears to have caused a GTF production level above that of the wild type.

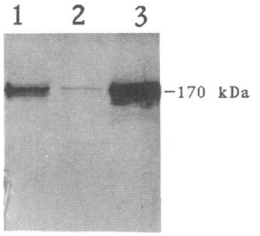

FIG. 5. GTF activity gel, showing differences in band intensity of culture supernatant samples. *S. gordonii* CH1 (lane 1), DS512 (lane 2), and DS512(pAM-S57) (lane 3) were grown to the same cell density; the supernatant was subjected to polyacrylamide gel electrophoresis and assayed for GTF activity as described in Materials and Methods. The bands represent relative GTF activity in the different strains.

DISCUSSION

We have identified a gene (*rgg*) on the *S. gordonii* Challis chromosome which positively regulates a GTF determinant, designated *gtfG*, located immediately downstream. A frameshift mutation in *rgg* resulted in a dramatic decrease in GTF synthesis, and the lesion was complemented in *trans* by a multicopy plasmid carrying *rgg*. In addition, when this plasmid chimera was present in wild-type Challis, GTF levels were much higher than normal. Since *rgg* was initially cloned on the basis of complementation of the Spp^- phenotype, it may also play a role in the altered GTF levels associated with the previously reported phase variation phenomenon (53). Moreover, mutation of the wild-type chromosomal *rgg* allele resulted in an Spp^- phenotype. Thus, the phase variation event involves a genetic "switch," altering *rgg* activity or expression of a determinant that can be suppressed by multiple copies (i.e., on plasmid DNA) of *rgg*. Further studies are needed to ascertain the mechanism of the switch.

The sequence data revealed two interesting inverted repeats. The first is an imperfect inverted repeat (IR-1) encompassing a 26-bp region between the putative −10 promoter site and the ribosome-binding site of *rgg*. This dyad symmetry is located in a position reminiscent of transcriptional repressor-operator sites (8, 21). It is conceivable that binding of a protein here would block transcription initiation of *rgg*. Two separate mutations, *gtf-20* and *gtf-30*, in *S. gordonii* Challis have recently been reported by Haisman and Jenkinson (17) to increase GTF expression 2.6-fold and 4.7-fold, respectively. Their evidence suggested that the mutations defined two different genetic loci. It is possible that these mutations map separately from the *rgg/gtfG* locus in a gene(s) whose product acts at IR-1 to block or reduce *rgg* transcription.

The second inverted repeat (IR-2) is positioned between *rgg* and *gtfG*, and it may play a role in both transcription and translation of *gtfG*. The inverted repeat may result in one of two RNA conformations. One resembles a factor-independent transcription termination signal (Fig. 6A). A run of U's following the structure is suggestive of a factor-independent signal (5). If transcription proceeds and the stem is increased by 6 bp, another more-stable RNA hairpin structure could form (Fig. 6B). Transcription may not terminate at this second structure owing to a lack of U's following the stem-loop, making it possible to continue into the *gtfG* gene. It may be significant that the *gtfG* ribosome-binding site lies within this hairpin structure when an *rgg/gtfG* transcript is produced. It is known that initiation of translation is severely reduced when a ribosome-binding site lies within an RNA secondary structure (16). Also, a process called translational coupling (16) can occur when an RNA hairpin containing the ribosome-binding site of a downstream gene is disrupted by translation of an upstream open reading frame. Disruption of the hairpin frees the ribosome-binding site for translational initiation of the downstream open reading frame. As has been postulated for lysozyme expression in bacteriophage T4 (50), translation of *rgg* to the base of the large RNA

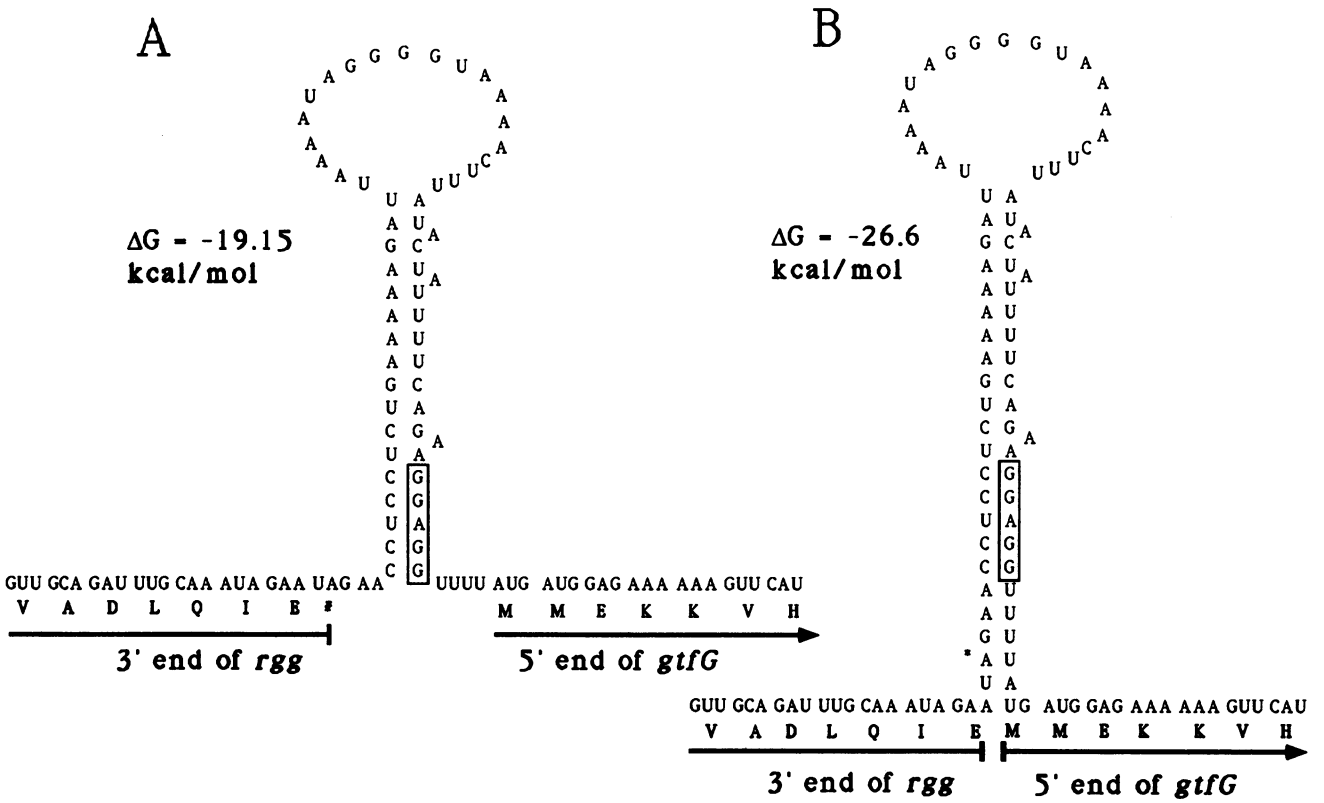

FIG. 6. Putative RNA secondary structures at the junction of the *rgg* and *gtfG* genes. The *rgg* stop codon UAG is indicated by an asterisk. Deduced amino acid sequences are in boldface type. The putative ribosome-binding site is boxed. The free energy of each structure is shown (ΔG). A possible factor-independent termination structure is seen (A), as well as a larger RNA hairpin structure (B).

stem-loop could disrupt the stem-loop, and the ribosome-binding site of *gtfG* would be available for initiation of translation. Conceivably, pausing of RNA polymerase caused by the longer stem would help to ensure that translation at the *rgg* terminus was close behind before transcription continued on.

Promoters in the bacteriophage T4 lysozyme (33) and *soc* (29) genes are located within DNA inverted repeats, so that transcription begins at points where translation-inhibitory RNA hairpins are unable to form; transcription of *gtfG* appears to be analogous to that of those genes. The presence of a transcriptional initiation site within the inverted repeat IR-2 would result in a *gtfG* transcript which lacks a translation-inhibitory RNA hairpin. Initiation of translation could then occur efficiently. In addition, it is conceivable that the *rgg* gene product interacts in or near this region to enhance transcription. Positive transcription-regulatory sites in *E. coli* tend to deviate from the −35 consensus sequences of sigma 70 promotors (8). The putative *gtfG* −35 element contains only three of six consensus nucleotides.

An obvious question is raised by these observations: what are the conditions that regulate the system? Since sucrose has been shown to influence *gtf* expression in *S. mutans* (23), and Tween 80 (6), sodium ion concentration (6), and growth conditions in general (57, 58) affect levels of extracellular GTF expression in streptococcal species, substances like these are good candidates. In some cases, *rgg* expression may be affected, which would then influence *gtfG* expression either transcriptionally or translationally. Until appropriate studies are done, one can only speculate on the significance of regulation of GTF in *S. gordonii*. However, the phase variation phenomenon is thought to play an important role, in that spontaneously arising Spp^- variants differ from Spp^+ cells in not being able to accumulate on saliva-coated hydroxyapatite beads during growth in the presence of sucrose (55). Perhaps this property would ultimately help enable the bacteria to relocate to an alternate niche.

We were not able to determine a possible mechanism of phase variation from the sequence analysis. There appear to be no obvious long runs of a particular nucleotide or contiguous repeat regions which indicate where frameshift mutations can occur. Frameshifting in such regions has been seen in other bacterial phase variation systems (35, 49). We were unable to identify DNA rearrangements associated with Spp^+ and Spp^- phase variants at the *rgg/gtfG* locus by Southern blot analysis with chromosomal DNA digested with a number of different restriction enzymes (data not shown). Therefore, we cannot yet implicate a DNA inversion (15) in this region, nor have we ruled out frameshift mutations for the phase variation phenomenon. Whatever the mechanism, it would appear to relate either to a reduced activity of *rgg* or to an alteration in a distal locus that can be suppressed in *trans* by additional copies of *rgg*.

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