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Molecular analysis of representative *Streptococcus gordonii* Spp phase variants reveals no differences in the glucosyltransferase structural gene, *gtfG*

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Streptococcus gordonii glucosyltransferase polymerizes sucrose to form glucans, which confer a hard, sucrose-promoted phenotype (Spp^+) to colonies on sucrose agar plates. The glucosyltransferase structural gene, *gtfG*, is positively regulated by the upstream determinant, rgg. Strain Challis undergoes a spontaneous, reversible phase variation between high (Spp^+) and low (Spp^-) levels of glucosyltransferase activity. Representative strains were examined to gain insights into the basis of glucosyltransferase phase variation. Western blots indicated that the level of glucosyltransferase activity was related to the amount of extracellular glucosyltransferase protein produced by Spp^- and Spp^+ strains. The nucleotide sequence of *rgg* and *gtfG* of the Spp^- strain CH97 was found to be identical to that of the Spp^+ parent, indicating that DNA differences in these regions are not the basis for glucosyltransferase phase variation. Indeed, ^{13}C-NMR spectroscopy suggested that glucans synthesized by strain CH97 glucosyltransferase were similar to those synthesized by glucosyltransferase of the Spp^+ parental strain, indicating a quantitative rather than qualitative change. However, one Spp^- strain, CH1C1, had a point mutation in *rgg*; replacement of the parent *rgg* with the CH1C1 allele resulted in decreased levels of glucosyltransferase protein and activity. The results indicate that glucosyltransferase phase variation can occur in more than one way, and suggest that glucosyltransferase regulation may involve distally located regulatory gene(s) that affect *rgg* and/or *gtfG* expression.

Key words: *Streptococcus gordonii*; oral streptococci; glucosyltransferase; phase variation; nucleotide sequence

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Streptococcus gordonii is a significant component of early, supragingival dental plaque (5) and a potential pathogen associated with infective endocarditis (18, 22). These bacteria have a single extracellular glucosyltransferase enzyme that polymerizes sucrose to make glucans with varying proportions of α1,6 and α1,3 linkages resulting in water-soluble and water-insoluble polymers (8, 10). Although the glucosyltransferase enzymes of the mutans streptococci have been extensively studied due to their potential role as virulence factors (14), little is known about the functions of glucosyltransferases of other oral streptococci.

S. gordonii strain Challis CH1 undergoes a spontaneous, reversible phase variation between high (Spp^+) and low (Spp^-) levels of glucosyltransferase activity (28). Phase variation is thought to provide bacteria with ecologically advantageous alternatives (20), which could be particularly important in the rapid, extreme changes of the oral environment. Although the *in vivo* role of

Table 1. Bacterial strains and plasmids used in these studies

	Relevant characteristics	Source or reference
Bacteria		
S. gordonii		
CH1	parental Challis strain, Spp^+	28
CH1A8	Spp^- phase variant of strain CH1	28
CH1B1	Spp^+ phase variant of strain CH1A8	28
CH1C1	Spp^- phase variant of strain CH1B1	28
CH1D2	Spp^+ phase variant of strain CH1C1	28
CH97	Spp^- phase variant of strain CH1	28
CHC10E	CH1 with chromosomally integrated pAM6203; erm^r	This study
CHC102	erm^s, Spp^- derivative of CHC10E	This study
CHC105	erm^s, Spp^- derivative of CHC10E	This study
CHC10H	erm^s, Spp^+ derivative of CHC10E	This study
E. coli		
DH5α	*recA1* cloning host strain	Bethesda Research Laboratories
Plasmids		
pVA891	*E. coli* vector, no gram-positive replication origin; em^r	15
pBluescript $KSII^+$	*E. coli* vector; Ap^r	Stratagene Cloning Systems
pAM6203	ca. 1.2-kb fragment carrying *rgg* of strain CH1C1 in pVA891	This study

glucosyltransferase phase variation is unknown, *in vitro* studies with *S. gordonii* Spp^+ and Spp^- strains suggest that these cells have different abilities to attach to and accumulate on various surfaces under different environmental conditions (30–32) and provide intriguing preliminary evidence that glucosyltransferase phase variation may allow differential colonization of oral sites. Additional phenotypic changes in cell surface properties that potentially could affect colonization, such as the ability of the cells to coaggregate or produce hemolysin, also undergo reversible phase variation in *S. gordonii* (11). Phase variation in one phenotype correlates with an increased probability of phase variation in additional phenotype(s) (11). The relationship between glucosyltransferase phase variation and variation in these other *S. gordonii* cellular phenotypes is unknown, and more than one mechanism may be involved.

The strain Challis glucosyltransferase structural gene, *gtfG*, is similar to other streptococcal *gtf* genes (35). After a conserved signal sequence, there is an approximately 600-bp region distinct for *S. gordonii gtfG*, followed by a conserved region encoding a putative catalytic site (6, 17). In the region encoding the carboxyl terminus there is a series of six direct repeats thought to be involved in glucan binding (4, 16). The open reading frame is followed by a region of dyad symmetry that may function in transcriptional termination (35). *gtfG* is positively regulated by the upstream gene, *rgg* (27); *rgg* is the only described regulatory determinant for a streptococcal *gtf* gene and is able to act in *trans* to increase glucosyltransferase activity in both Spp^+ and Spp^- strains (27). Southern hybridization analyses indicate that *rgg*- and *gtfG*-like determinants are present in other early plaque organisms including other *S. gordonii* strains as well as *Streptococcus sanguis* and *Streptococcus oralis* (34).

No major DNA rearrangements in *rgg/gtfG* and their flanking regions have been detected in phase variant strains (27). The level of glucosyltransferase activity varies among different Spp^+ and Spp^- strains (28), suggesting that phase variation is not due to an "on-off" switch. The purpose of the present studies was to further characterize representative phase variant strains and to examine the genes known to be involved in glucosyltransferase expression in these strains in an attempt to better understand the basis for glucosyltransferase phase variation at the DNA level.

Material and methods

Bacterial strains and plasmids

S. gordonii CH1 (28) was the parental strain Challis. A series of glucosyltransferase phase variants (28) that were sequentially derived from the Spp^+ parent strain CH1 [strain CH1A8 (Spp^- variant of strain CH1), CH1B1 (Spp^+ revertant of strain CH1A8), CH1C1 (Spp^- variant of strain CH1B1) and CH1D2 (Spp^+ revertant of strain CH1C1)] as well as the independently derived, reversible Spp^- variant CH97 were the phase variant strains chosen for examination. The relevant characteristics and references for bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions

S. gordonii strains were grown in Todd Hewitt medium (Difco Laboratories, Detroit, MI) at 37°C in a candle jar or anaerobically (Gas Pak Plus Anaerobic System, BBL Microbiology Systems, Cockeysville, MD). Strains with integrated pVA891 (15) or pAM6203 were grown with 5 μg/ml erythromycin. Spp colony morphology was determined as hard (Spp^+) or soft (Spp^-) on Todd Hewitt 3% sucrose agar plates (28). For *in vitro* glucan synthesis and analysis, cells were grown in defined FMC medium (29) in an anaerobic chamber (Coy Industries, Jackson, MI).

Escherichia coli DH5α strains with cloned inserts in pBluescript were selected on LB (2) agar plates containing 100 μg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside and 12 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. *E. coli* DH5*a* strains containing recombinant pBluescript plasmids were grown in LB medium with 100 μg/ml ampicillin. DH5α strains carrying pVA891 or pAM6203 were grown in 200 μg/ml erythromycin.

DNA isolation and manipulation

Streptococcal chromosomal DNA was prepared by a modified Sarkosyl lysis procedure using mutanolysin and lysozyme (34). Double-stranded DNA subclones or polymerase chain reaction fragments were digested with appropriate restriction enzymes, ligated with T4 DNA ligase into the multiple cloning region of pBluescript $KSII^+$, and transformed into *E. coli* DH5α by the $CaCl_2$ method (2). Plasmid DNA from *E. coli* was prepared by Qiagen purification columns (Qiagen, Chatsworth, CA) according to the manufacturer's directions. *S. gordonii* strains were transformed by a

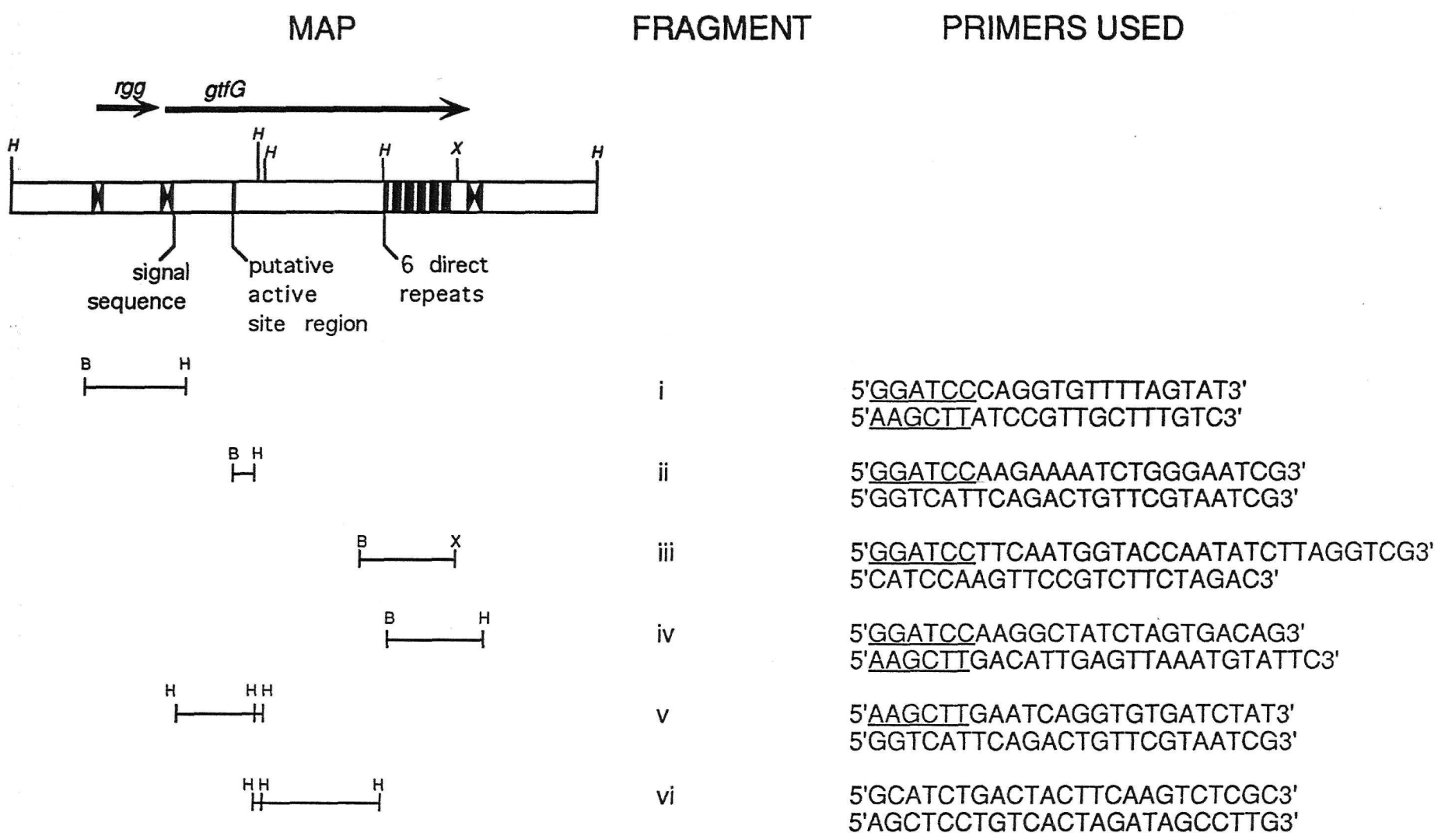

Fig. 1. Polymerase chain reaction-generated subclones used for determining the nucleotide sequence of Spp$^+$ and Spp$^-$ phase variant strains. The GenBank accession numbers for the strain CH1 *rgg* (27) and *gtfG* (35) sequences are M89776 and U12643, respectively. The map shows the relative position of each fragment. The heavy arrows above the map indicate the *rgg* and *gtfG* open reading frames. Regions of dyad symmetry are indicated by inverted arrows. Relevant regions of *gtfG* are described. The oligonucleotide primers listed for fragments i, ii, iii, and iv were used in polymerase chain reaction with chromosomal template DNA from the phase variant strains CH1A8, CH1B1, CH1C1, CH1D2, and CH97. Primers either had an engineered flanking *Bam*HI or *Hin*dIII site (underlined) or else included, or were immediately upstream from, a convenient restriction site for cloning the double-stranded polymerase chain reaction product into the *Bam*HI (B), *Hin*dIII (H) or *Xba*I (X) sites of pBluescript KSII$^+$. In some cases partial, rather than complete, *Hin*dIII digestion of the polymerase chain reaction fragments was necessary for cloning. To complete the entire sequencing of *gtfG* and its flanking regions in an Spp$^-$ phase variant strain, fragments v and vi were generated from strain CH97 chromosomal DNA.

modified method of Lawson & Gooder (13) using horse serum (27).

Polymerase chain reaction

Double-stranded polymerase chain reaction products were obtained with a GeneAmp polymerase chain reaction kit in a 480 DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA) using AmpliTaq enzyme as previously described (35). Custom oligonucleotide primers were synthesized at the DNA Core Facility (University of Michigan, Ann Arbor).

Construction of subclones of *rgg* and *gtfG* and their flanking regions from phase-variant strains

Chromosomal DNA samples from Spp$^+$ and Spp$^-$ phase variant strains were used as templates in polymerase chain reaction reactions with oligonucleotide primers shown in Fig. 1. Primers were designed to amplify areas of interest in *rgg*, *gtfG* and their flanking regions. Primers either had engineered 5′ restriction sites or else were designed to anneal to the template immediately 5′ to a convenient restriction site so that digestion of the amplified fragment would facilitate ligation into the multiple cloning region of pBluescript KSII$^+$. Each polymerase chain reaction gave only one product. To guard against mistakes due to misincorporation of nucleotides during polymerase chain reaction, three independent products from each strain were sequenced.

Nucleotide sequence determination and analysis

Double-stranded DNA sequencing was done by a modification (35) of the dideoxy chain termination sequencing method (21) using M13 forward and reverse primers for the pBluescript cloning vector and custom oligonucleotide primers (University of Michigan DNA Core Facility) as necessary. To determine the sequence of the *gtfG* carboxyl-terminal direct repeat regions that had approximately 600-bp regions without unique sites for custom primers (35), a cycle sequencing method was used at the University of Michigan DNA Core Facility. Templates were sequenced with a PRISM-Ready Reaction Dye Deoxy Terminator Sequencing kit (Applied Biosystems, Foster, CA) using AmpliTaq enzyme according to the manufacturer's directions. Samples were run in a model 373 DNA Stretch Sequencer with analytical software (Applied Biosystems). Both strands of DNA were sequenced.

Individual DNA sequences were stored in AssemblyLign (International Biotechnologies, New Haven, CT) and compared and analyzed with the IBI

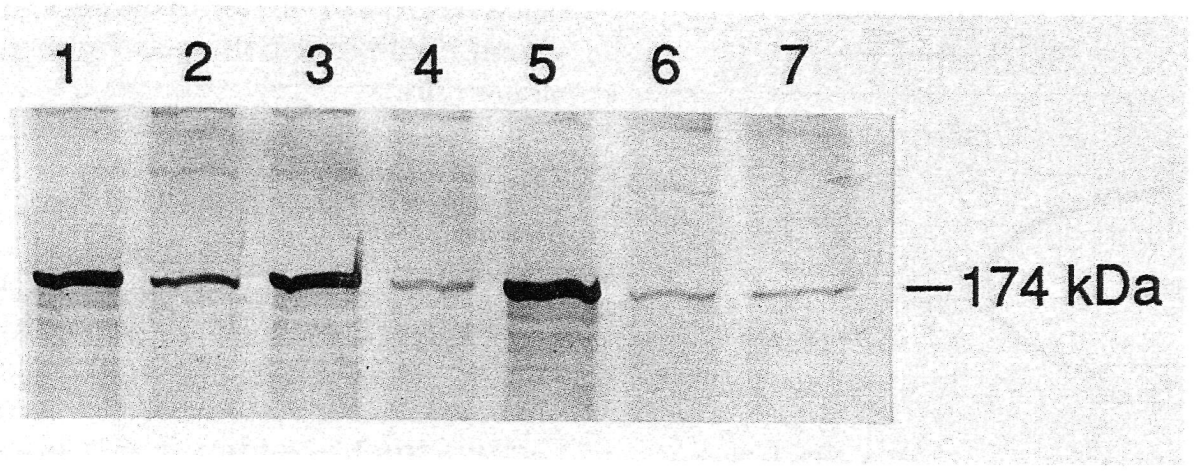

Fig. 2. Western blot analyses of *S. gordonii* strains show that the level of glucosyltransferase activity in each strain is related to the level of extracellular glucosyltransferase antibody-reactive protein. Cultures were grown in TH broth to the same phase (OD_{520}=1.2) and cell-free supernatants were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes and GTF-I antibody-reactive proteins were detected. The parental strain CH1 is shown in lane 1. The sequentially derived phase variants are shown in lane 2) the Spp^- strain CH1A8; 3) the Spp^+ strain CH1B1; 4) the Spp^- strain CH1C1; 5) the Spp^+ strain CH1D2. The independently-derived, reversible Spp^- strain CH97 is shown in lane 6. Lane 7 shows strain CHC102 (see text).

MacVector software program (International Biotechnologies).

Determination of glucosyltransferase activity

For relative comparisons of glucosyltransferase activity, cultures were grown to the same optical density at 520 nm and the cell-free supernatants were subjected to SDS-PAGE. The relative extracellular glucosyltransferase activity of each strain was determined by densitometric scan of glucosyltransferase activity gels as previously described (27).

Southern hybridization analysis

DNA was digested with appropriate restriction enzymes, electrophoresed on 0.7% agarose gels and transferred to Hybond-N (Amersham) membrane based on the method of Southern (2). Probe DNA was labelled with digoxigenin-dUTP, hybridized to the membrane under stringent conditions (2) and detected by chemiluminescence with the Genius System (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's directions.

Analysis of glucosyltransferase antibody-reactive proteins using Western blots

S. gordonii strains were grown in Todd Hewitt broth to the same mid-to-late log growth phase (optical density at 520 nm =1.2). After removal of the cells by centrifugation at 1000×*g*, supernatants were concentrated 10-fold in Centricon-50 concentrators (50 kDa cutoff; Amicon, Beverly, MA) and equal volumes loaded on 7.5% SDS-acrylamide gels (12). After electrophoresis, proteins were transferred with a transblot cell (Bio-Rad Laboratories, Hercules, CA) onto polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA) in 10 mM 3-cyclohexylamino-1-propane sulfonic acid buffer, pH 11.0. Proteins were detected with polyclonal anti GTF-I rabbit antiserum raised against the product of *S. mutans* GS5 *gtfB* (26) (a gift from H. K. Kuramitsu). Based on amino acid sequences deduced from the nucleotide sequences (26, 35), the GTF-I protein is 51.5% identical to *S. gordonii* CH1 glucosyltransferase as determined by the GCG Wisconsin Gap program for protein similarity (9). The anti-GTF-I antiserum was sufficiently cross-reactive to detect the *S. gordonii* glucosyltransferase; control blots using increasing amounts of partially purified strain CH1 glucosyltransferase confirmed that the intensity of the bands on the Western blots was proportional to the amount of *S. gordonii* supernatant extract present. Primary antibody binding was detected with goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Bethesda Research Laboratories, Gaithersburg, MD) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma Chemical Co., St. Louis, MO).

In vitro glucan synthesis

For glucan analysis, 20-ml cultures of strain CH1 and strain CH97 were grown anaerobically in FMC with 1 mM phenylmethylsulfonyl fluoride until late log phase (optical density at 520 nm =1.8). Cell-free culture supernatants were concentrated 20-fold in Centricon-100 concentrators (100 kDa cutoff; Amicon). This partially purified enzyme was added to 50 ml of filter-sterilized substrate solution consisting of 5% sucrose, 1 mM phenylmethylsulfonyl fluoride in 0.05 M sodium phosphate buffer, pH 6.8. After gentle rotation at 37°C for 60 h, the glucans were precipitated with 3 volumes of 95% ethanol at –20°C and collected by centrifugation at 16,000 × *g*. The glucans were washed with water and reprecipitated three times. The final washed glucan products were dried in a Speedvac concentrator (Savant Instruments, Farmingdale, NY).

Nuclear magnetic resonance analysis of glucans

Glucans synthesized *in vitro* were examined by ^{13}C-NMR spectroscopy at the University of Michigan Core Facility. Spectra of saturated glucans in dimethyl-d6-sulfoxide were obtained in a GE Omega 500 spectrometer at 124 MHz in a 10-mm tube with a probe temperature of 75°C. 16,000 data points over a spectral width of 26,315 Hz for 60,000 scans were collected. Relaxation time was 2.0 s and digital resolution was 3.2 Hz. Dimethyl-d6-sulfoxide was used as the internal reference (40 ppm). Peak assignments for carbons of glucose moieties in α1,6 and α1,3 linkages were based on data of Colson et al. (3) and Seymour et al. (23).

Adhesion to hydroxyapatite

The adhesion of cells to hydroxyapatite and saliva-coated hydroxyapatite beads was performed as described previously (32). Briefly, cells were grown anaerobically to late-log phase in the presence of 2 μCi/ml of [^{3}H]-thymidine, washed and resuspended to a concentration of approximately 1 × 10^8 cells/ml in buffered KCl (1). One-ml volumes of radiolabelled cells were incubated for 3.5 h at 10 rpm with 10 mg of hydroxyapatite beads (BDH, Poole, UK) that had either

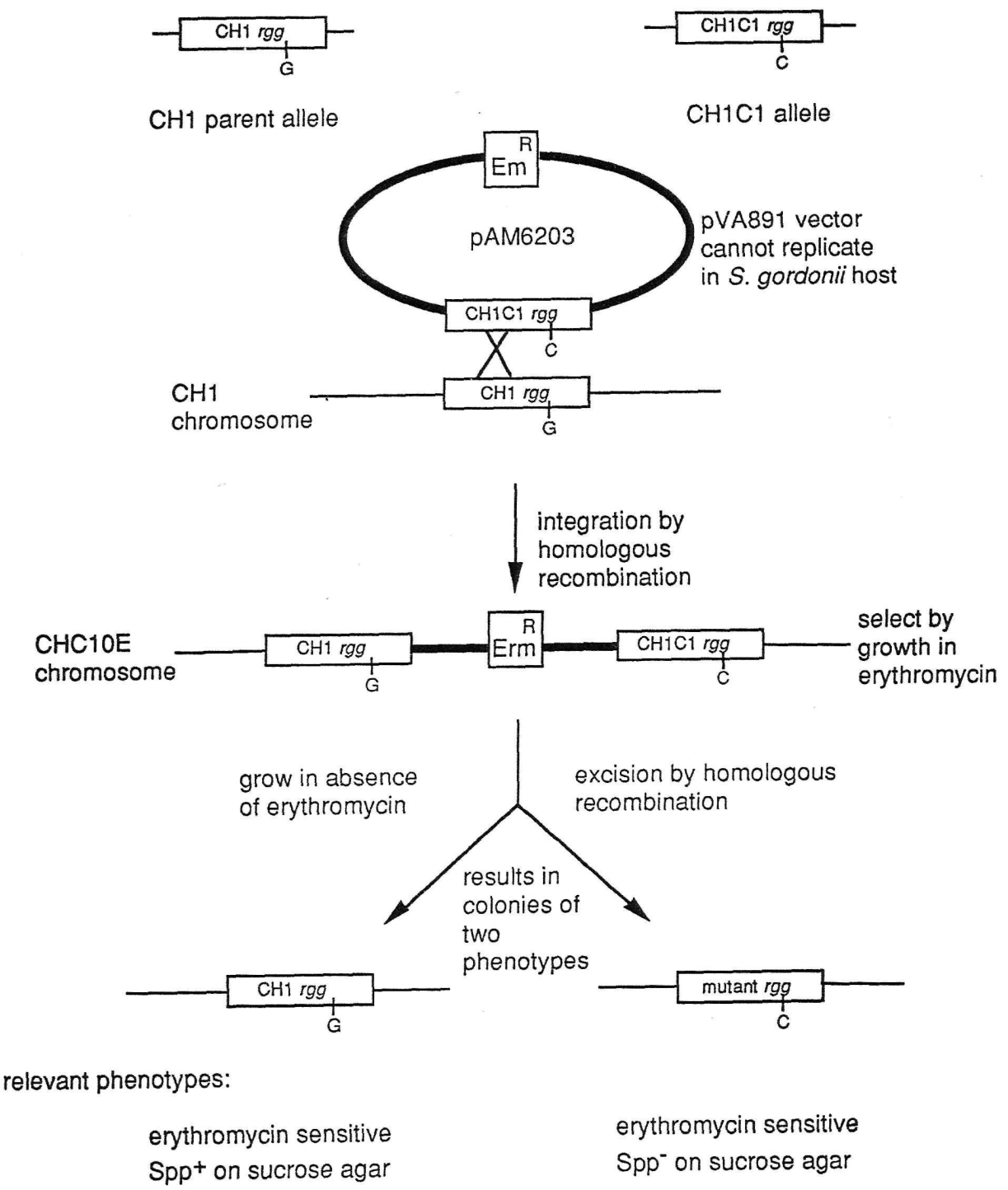

Fig. 3. Diagram of the construction of strains CHC102 and CHC105, the Spp⁻, erythromycin-sensitive strains that contains the point mutation found in the *rgg* gene of strain CH1C1, and strain CHC10H, the Spp⁺ erythromycin-sensitive strain with the parental *rgg* sequence.

been buffer-equilibrated or coated with human saliva. Supernatants containing unattached bacteria were removed; the beads containing attached bacteria were washed and counted in a scintillation counter. Radiolabel counts were correlated with bacterial numbers, and adhesion was expressed as the number of attached bacteria per 10 mg of beads. Statistical comparisons between strains were done by a one-tailed Student *t* test.

Hemolysin production

β-hemolysin production by bacterial strains was detected on agar medium containing 4% washed horse blood in 1% LabLemco, 1% peptone, 0.5% NaCl (Oxoid, Unipath, Nepean, Ontario), pH 7.3, as previously described (11). Plates were incubated anaerobically and strain CH1, which does not produce β-hemolysin, and strain CH1C1, which is β-hemolytic under these conditions, were used as negative and positive controls, respectively.

Results

Spp⁻ strains produce less extracellular glucosyltransferase antibody-reactive protein than Spp⁺ strains

Western blot analyses showed that a basis for the decreased glucosyltransferase activity in Spp⁻ phase variant strains was their decreased amount of extracellular glucosyltransferase protein. The Spp⁺ strains (Fig. 2, lanes 1, 3 and 5) had approximately 3- to 5-fold more glucosyltransferase antibody-reactive protein than the Spp⁻ strains (Fig. 2, lanes 2, 4 and 6), and the relative intensities of the antibody-reactive protein bands correlated to the intensities of glucan bands on activity gels for these strains (28).

Comparison of glucan products of Spp⁺ and Spp⁻ strains

^{13}C-NMR spectra of glucans produced by partially purified glucosyltransferases from the parental Spp⁺ strain CH1 and the Spp⁻ strain CH97 were similar (data not shown) to previously published ^{13}C-NMR spectra of glucans produced by strain Challis (10). The peak positions and ratios of areas under the peaks representing the carbons in the C1, C3 and C6 positions of the glucose moieties in the glucans were similar in spectra from both strains, indicating that the glucans made by glucosyltransferase enzymes from strain CH1 and CH97 were similar. Thus, the glucans produced *in vitro* by strains CH1 and CH97 appear to differ primarily in quantity rather than quality.

Comparison of nucleotide sequences of *rgg* and *gtfG* regions of Spp phase variants

To examine glucosyltransferase phase variation at the DNA level, the nucleotide sequences of a series of sequentially derived phase variant strains (CH1A8, CH1B1, CH1C1 and CH1D2) and the independently derived Spp⁻ strain CH97 were examined. Since preliminary evidence suggested that glucosyltransferase phase variation can occur in more than one way (33), regions with the seemingly greatest potential for controlling *gtfG* expression were examined in all five phase variant strains (Fig. 1). These regions included: the upstream regions and dyad symmetry regions at the beginning of *rgg* and *gtfG*, which could affect nucleic acid secondary structure and could act as potential binding sites for regulatory factors; the *rgg* regulatory determinant for *gtfG* expression; the signal sequence of *gtfG*, which encodes the region responsible for transport of the functional enzyme; the putative active site region; the carboxyl terminal repeats involved in glucan binding; and the *gtfG* stop codon and downstream region of dyad symmetry. In addition, the entire *gtfG* gene and its flanking regions were examined in strain CH97, which has the highest reversible frequency for glucosyltransferase phase variation (10^{-3} in both directions) (28).

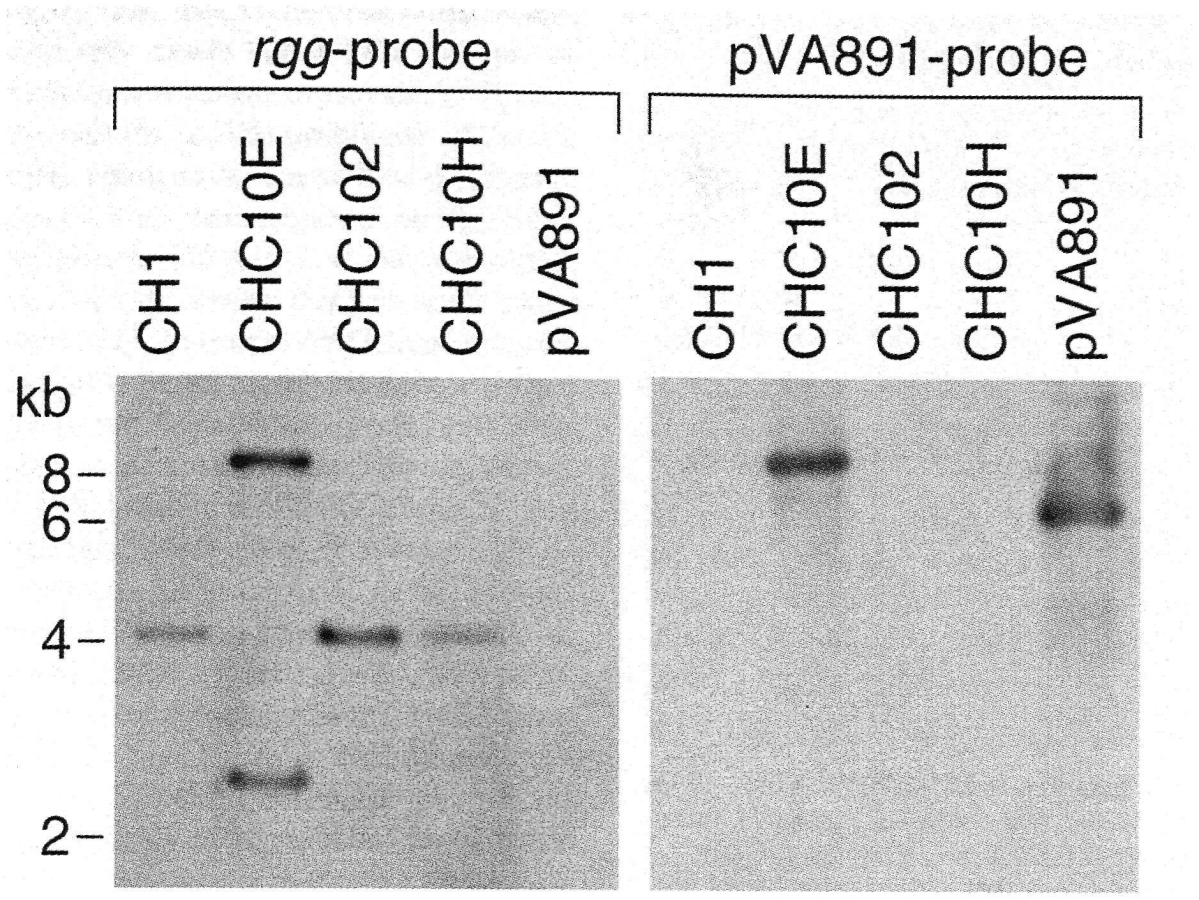

Fig. 4. Southern hybridization analysis of *Hin*dIII-digested chromosomal DNA from strains CH1, CHC10E, CHC102 and CHC10H probed with an internal fragment of *rgg* (34) (left panel) and pVA891 (right panel). DNA size standards (Bethesda Research Laboratories) ran as marked.

Table 2. Adhesion to hydroxyapatite and saliva-coated hydroxyapatite beads

Strain	Substratum	
	hydroxyapatite	saliva-coated hydroxyapatite
CH1	7.68±0.85	4.92±0.50
CH1C1	17.34±0.53	15.73±0.15
CHC102	8.17±0.40	5.27±0.07
CHC105	8.12±0.56	5.10±0.50

Number of washed, radiolabelled bacteria $\times 10^6$ (±SD) attached to hydroxyapatite and saliva-coated hydroxyapatite beads after 3.5 hours incubation. Tests were done in duplicate and repeated twice. The percentage recovery of total radioactive counts ([number of counts in the supernatant plus number of counts on the beads divided by the initial washed cell counts/ml]×100) for all tests ranged from 85% to 98%.

No differences from the parental sequence were found in the regions of nucleotide sequences of *gtfG* examined in any of the phase variant strains; nor were any differences seen in the regions flanking *rgg* and *gtfG,* indicating that changes in these regions are not responsible for phase variation in these strains. The nucleotide sequence of the *rgg* structural gene was also the same as the strain CH1 parent sequence in strains CH1A8, CH1B1, CH1D2 and CH97 indicating that differences in the known *gtfG* regulatory determinant, *rgg*, were not responsible for variable expression of *gtfG* in these strains. However, strain CH1C1, an Spp^- variant of strain CH1B1, had a point mutation in the *rgg* structural gene.

Construction and characterization of strains with the *rgg* of strain CH1C1

In the *rgg* of strain CH1C1 a guanine was replaced by a cytosine, resulting in a change at amino acid number 271 (27) from an aspartate (negative charge) to a histidine (positive charge) residue. To see whether this point mutation was related to the Spp^- colony morphology, the mutation was introduced into the Spp^+ parental strain CH1 by homologous recombination in a strategy previously described (27) (Fig. 3). Briefly, a clone containing the sequenced *rgg* gene of strain CH1C1 (fragment i from strain CH1C1, Fig. 1) was digested with *Bam*HI and *Hin*d III, electrophoresed on 0.7% agarose and the 1.2-kb fragment eluted. This fragment was then directionally cloned into the *Bam*HI and *Hin*dIII sites of the vector pVA891 (15). The resulting plasmid, pAM6203 (Fig. 3), was transformed into the parental strain CH1 and transformants were selected on erythromycin agar. Since pVA891 does not have a gram-positive origin of replication, only those colonies with pAM6203 integrated into the chromosome would be expected to grow under these conditions. The erythromycin-resistant transformants theoretically carried both a CH1 parental *rgg* gene and a CH1C1 mutated *rgg* gene (Fig. 3).

An erythromycin-resistant transformant colony was chosen and designated CHC10E. The insertion of pAM6203 into the *rgg* gene of strain CHC10E was confirmed by Southern hybridization analysis (Fig. 4) using probes for *rgg* (34) and pVA891. This strain was then grown in the absence of erythromycin so that the integrated vector could be lost by recombination of the flanking homologous DNA, theoretically resulting in both strains with the parental *rgg* and strains with the CH1C1 *rgg*. As expected, after selection for erythromycin sensitivity, both Spp^+ and Spp^- strains were recovered. An Spp^- colony designated CHC102 and an Spp^+ colony CHC10H were chosen for further analysis. Southern hybridization analysis of these strains (Fig. 4) indicated that each contained only one copy of *rgg* and did not contain the integrated pVA891 plasmid. Nucleotide sequence analysis confirmed that the *rgg* gene of strain CHC102 was identical to that of strain CH1C1. An additional erythromycin sensitive, Spp^- strain CHC105 also had the CH1C1 *rgg* sequence. Furthermore, nucleotide sequence analysis of strain CHC10H, an erythromycin sensitive, Spp^+ derivative of strain CHC10E had the parental *rgg* sequence.

The CH1C1 *rgg* allele is related to decreased glucosyltransferase activity

Glucosyltransferase activity gels showed that strain CHC102 had glucosyltransferase activity levels similar to that of strain CH1C1 (Fig. 5). Western blot analyses (Fig. 2, lane 7) indicated that like strain CH1C1, strain CHC102 had low levels of extracellular glucosyltransferase protein. These results imply that the point mutation in the *rgg* determinant of strain CH1C1 was related to the Spp^- colony morphology and low level of extracellular glucosyltransferase protein. However, since the Spp^- strains CH1A8 and CH97 had the parental *rgg* sequence, a change in *rgg* was not the basis for decreased glucosyltransferase activity in all Spp^- strains.

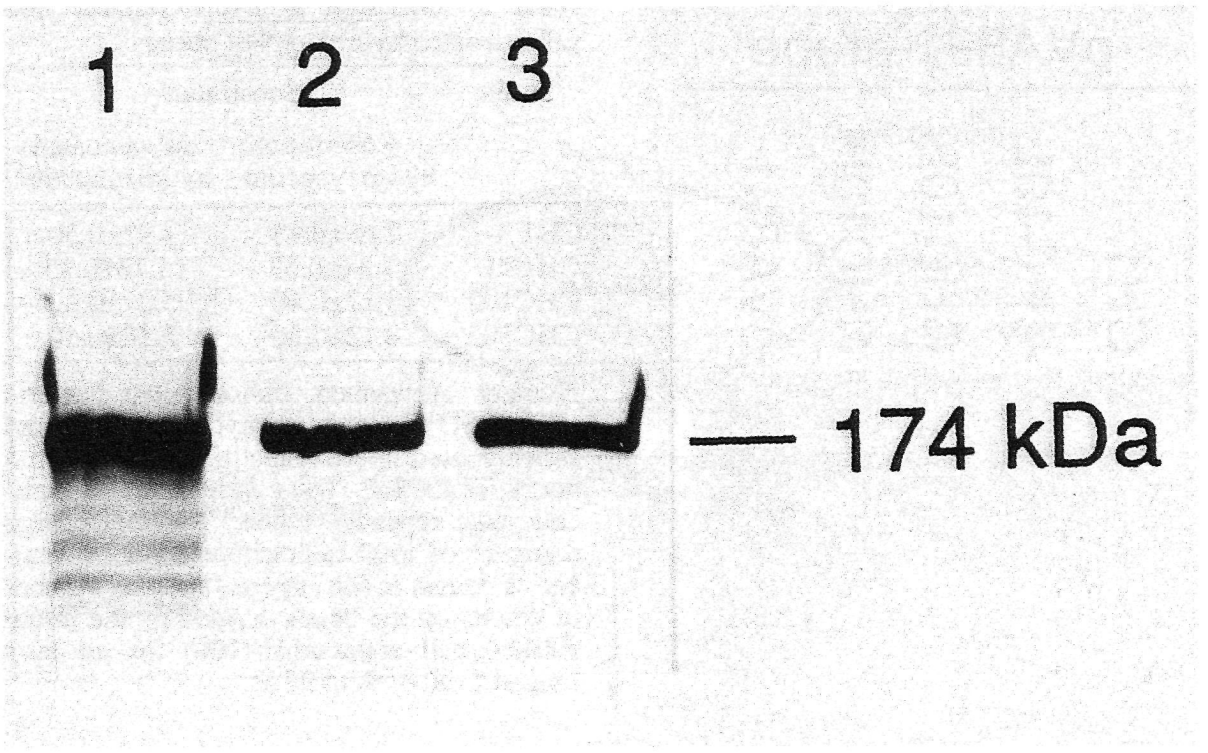

Fig. 5. Glucosyltransferase activity gel of the same preparations used for Western blots in Fig. 2 shows the level of glucosyltransferase activity of the parental strain CH1 (lane 1), the decreased level of activity of the Spp⁻ phase variant strain CH1C1 (lane 2) and the similarly low activity of strain CHC102 (lane 3), the derivative of strain CHC10E with the same point mutation in *rgg* as strain CH1C1. Strain CHC10H, the strain CHC10E derivative with the parental *rgg* sequence, had a glucosyltransferase activity level similar to that of strain CH1 (data not shown).

Although strains CHC102 and CHC105 had the *rgg* point mutation and Spp⁻ colony type of strain CH1C1, they differed from strain CH1C1 in two previously described phase variable phenotypes (11, 32). Strain CH1C1 has shown increased adhesion to saliva-coated hydroxyapatite compared with the Spp⁺ strains CH1, CH1B1 and CH1D2 (32). Strain CH1C1 also produces a β-hemolysin that is not produced by the parental strain (11). However, whereas CH1C1 showed increased attachment to both hydroxyapatite or saliva-coated hydroxyapatite beads ($P < 0.002$ and $P < 0.001$, respectively; Table 2), neither strain CHC102 nor CHC105 attached to either substratum significantly more than the parent (Table 2). Furthermore, neither CHC102 nor CHC105 produced β-hemolysin on washed blood agar plates. Thus, the nucleotide change in *rgg*, which caused decreased glucosyltransferase activity, was not responsible for these additional phenotypic changes in strain CH1C1.

Discussion

The glucosyltransferase of *S. gordonii* is unique in several ways. In addition to being the only glucosyltransferase with an identified regulatory determinant, *rgg* (27), it is, to our knowledge, the only oral streptococcal glucosyltransferase reported to undergo a phase variation phenomenon. It was previously shown that the spontaneous Spp⁻ phase variant strains have less glucosyltransferase activity than the Spp⁺ strains (28); the Western blot analysis presented here indicates that this decreased activity is a quantitative difference due to decreased amounts of extracellular glucosyltransferase protein in the Spp⁻ strains. The mobility of active glucosyltransferase proteins on SDS 6% polyacrylamide gels stained for glucosyltransferase activity (28; unpublished results) and the location of glucosyltransferase antibody-reactive proteins in immunoblots suggested that the five phase variant strains examined in this study had glucosyltransferase enzymes of the same molecular weight. These results did not preclude changes in enzyme size that were too small to be noted by gel electrophoresis. However, nucleotide sequence analysis of the region encoding the glucosyltransferase amino terminus as well as the region encoding the carboxyl terminus and downstream region in five phase variant strains confirmed that the size of the *gtfG* open reading frame in these strains was identical. Indeed, all the regions of *gtfG* examined in these strains were identical.

The mechanisms underlying the decreased amounts of extracellular glucosyltransferase protein and activity appear to differ among the Spp⁻ strains examined in this study. Phase variation events are known to occur in a number of ways, including DNA inversions, frameshifts and/or recombinations (19). Although no changes had been found previously in the *rgg/gtfG* region of phase variants by Southern analysis of chromosomal DNA digested with various restriction enzymes (27), it was possible that changes occurred but were simply not detected. Indeed, examination of strain CH1C1 indicated that a point mutation in the *gtfG* regulatory gene, *rgg*, had occurred. This point mutation in *rgg* was not present in either strain CH1A8 or CH1B1 from which strain CH1C1 was sequentially derived. Furthermore, it was not present in strain CH1D2, the Spp⁺ strain reported to be a revertant of CH1C1 (28). Although the probability of the exact nucleotide reversion occurring in strain CH1D2 would seem extremely low, this region of *rgg* may be a hot spot for mutations and/or our selection conditions of plating on sucrose agar picked up this rare mutation. Alternatively, there may have been an error in determining the lineage when these strains were originally isolated (28). However, the identical biochemical profiles and DNA restriction patterns (unpublished data) of strains CH1B1, CH1C1 and CH1D2 and the parental strain CH1 confirm that CH1C1 is an Spp⁻ derivative, and CH1B1 and CH1D2 are Spp⁺ derivatives of the parental strain CH1. Examination of strain CHC102, which contained an *rgg* allele with the same point mutation found in the *rgg* of CH1C1, suggested that this point mutation conferred an Spp⁻ colony morphology and decreased the level of extracellular glucosyltransferase; these results confirm the importance of *rgg* in glucosyltransferase regulation. However, the point mutation in *rgg* did not confer the changes in adhesive and hemolytic phenotypes that occur in strain CH1C1 (11) to any of the strain CHC10E derivatives examined. This finding supported previous studies (11) that suggested that phase variation in multiple phenotypes of *S. gordonii* is not related solely to *rgg* and probably involves more than one mechanism.

The basis of glucosyltransferase phase variation in strains CH1A8, CH1B1, CH1D2 and CH97 is still undetermined. Nucleotide sequence analysis

of *rgg* and *gtfG* subclones from Spp^+ and Spp^- strains showed no changes in regions known to be involved in enzyme function. The possibility of nucleotide changes in regions of *gtfG* that were not sequenced in strains CH1A8, CH1B1 and CH1D2 cannot be ruled out. Specific amino acid changes in *S. mutans* glucosyltransferases have been shown to influence the type of glucan produced (25). However, the ^{13}C-nuclear magnetic resonance data indicated that the glucans produced *in vitro* by the parental strain CH1 and the Spp^- strain CH97 were similar. Although nuclear magnetic resonance cannot accurately quantify the proportion of glucose moieties in each linkage within a glucan, since the samples were run under identical conditions, the similar peak positions and ratios of the areas under each peak can be compared (24). The data suggest that, at least for strain CH97, phase variation appears to affect primarily the quantity rather than the quality of the glucosyltransferase. Nucleotide sequence analysis confirmed that the entire *rgg/gtfG* and flanking regions in strain CH97 were identical to those of the parental strain. However, these *in vitro* results do not preclude the possibility that, *in vivo*, other factors could influence both the level of glucosyltransferase expressed and glucan products made by Spp^+ and Spp^- strains. These could include post-translational modifications of the enzyme and/or factors that affect the level of glucosyltransferase translocated across the cell membrane, as has been postulated to occur in *Streptococcus salivarius* (7). Furthermore, other, as yet undescribed, DNA region(s) may be responsible for the differential expression of *gtfG* and a basis for glucosyltransferase phase variation. Indeed, two distinct, unlinked genetic loci have been reported to increase glucosyltransferase activity in *S. gordonii* Challis (10). Both *rgg* and *gtfG* are preceded by regions of dyad symmetry that have been previously hypothesized to function in regulation of gene expression (27). Distally located genes might encode *trans*-acting factors that act at the *rgg* and/or *gtfG* loci.

The present studies indicate that phase variation involves primarily quantitative rather than qualitative changes in glucosyltransferase, which can occur without nucleotide changes in the structural gene, *gtfG* or its known regulatory gene, *rgg*. These results provide an essential basis for future studies aimed at understanding the complex genetic and environmental interactions that regulate *S. gordonii* glucosyltransferase.

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