Changes in the Carboxyl-Terminal Repeat Region Affect Extracellular Activity and Glucan Products of *Streptococcus gordonii* Glucosyltransferase

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Glucans produced by the glucosyltransferase (GTF) of *Streptococcus gordonii* confer a hard, cohesive phenotype (Spp⁺) on colonies grown on sucrose agar plates. *S. gordonii* strains with specific mutations in the region of *gtfG* that encodes the GTF carboxyl terminus were characterized. In the parental strain Challis CH1, this region included a series of six direct repeats thought to function in glucan binding. The spontaneous mutant strain CH107 had a 585-bp deletion resulting in the loss of three internal direct repeats. Insertional mutagenesis was used to construct strain CH2RPE, which had the parental repeat region but was missing 14 carboxyl-terminal amino acids. The similarly constructed strain CH4RPE had an in-frame addition of 390 nucleotides encoding two additional direct repeats. Although strains CH1, CH2RPE, and CH4RPE all had similar levels of extracellular GTF activity, strain CH107 had less than 15% of the parental activity; however, Western blots (immunoblots) indicated that the amounts of extracellular GTF protein in all four strains were similar. ¹³C NMR analyses indicated that partially purified GTFs from the Spp⁺ strains CH1, CH2RPE, and CH4RPE all produced glucans with similar ratios of α 1,6 and α 1,3 glucosidic linkages, whereas the Spp[−] strain CH107 GTF produced primarily α 1,6-linked glucans. Transformation of strain CH107 with pAMS57, which carries the *gtfG* positive regulatory determinant, *rgg*, increased the amount of GTF activity and GTF antibody-reactive protein ca. fivefold but did not confer a hard colony phenotype on sucrose agar plates, suggesting that the type of glucan product affects the sucrose-promoted colony phenotype.

The early-colonizing dental plaque organism *Streptococcus gordonii* (7) has a single, primer-independent (11) glucosyltransferase (GTF) enzyme that hydrolyzes sucrose and polymerizes the glucose moieties to form glucans with different proportions of both α 1,3 and α 1,6 glucosidic linkages (12, 14). Biochemical studies (11) and nucleotide sequence analysis (42) indicate that the native enzyme is ca. 174 kDa. In addition to the native enzyme, lower-molecular-mass forms of this enzyme are seen with a major band at ca. 156 kDa (4, 12, 38) presumably as a result of endogenous proteolytic activity (11).

S. gordonii undergoes a reversible phase variation between high and low levels of GTF activity (36). The glucans formed by the higher (parental) levels of GTF make colonies hard on sucrose agar plates; colonies with this phenotype are designated Spp⁺ (sucrose-promoted phenotype positive). Colonies with lower levels of GTF activity are soft on sucrose agar plates and designated Spp[−]. The *S. gordonii* GTF structural gene, *gtfG*, is regulated by the only described *gtf* genetic regulatory determinant, *rgg* (35). *rgg* can act in *trans* to increase the level of GTF activity in both Spp⁺ and Spp[−] reversible phase variants, resulting in increased colony hardness in strains of both types and conferring an Spp⁺ colony morphology on the latter (16, 35).

Although GTF enzymes of other oral streptococci such as the mutans streptococci and *Streptococcus salivarius* have been the focus of much study, these species have multiple GTFs within one organism (28). Their enzymes have been classified

by their primary products; GTF-S enzymes make glucans with predominantly α 1,6 linkages, whereas GTF-I and GTF-SI enzymes make glucans with predominantly α 1,3 linkages. The different GTF enzymes and their products can interact with each other, making interpretation of results difficult. The presence of a single GTF which makes glucans with both α 1,3 and α 1,6 glucosidic linkages in *S. gordonii* obviates some of these difficulties and facilitates interpretation of genetic and functional studies because they can be performed in a parental background.

The nucleotide sequence of *gtfG* has been determined (42) and found to be similar to other streptococcal *gtf* genes. A conserved signal sequence is followed by a ca. 600-bp region containing both conserved sequences and sequences unique for *gtfG*. Like other streptococcal *gtf* genes, *gtfG* has a conserved region encoding a putative catalytic active site for sucrose hydrolysis (8, 23), and the carboxyl-terminal region has a series of direct repeats. These direct repeats have been implicated in glucan binding (6, 24) and are similar to direct repeats found in the carboxyl terminus of proteins of other gram-positive organisms. This family of ligand-binding proteins (46) includes *Clostridium difficile* ToxA and ToxB (43), a *Streptococcus mutans* glucan-binding protein (3), and lysins from *Streptococcus pneumoniae* and its bacteriophages (9).

The carboxyl-terminal repeats have been classified in several ways on the basis of sequence similarities (10, 27, 44). The numbers and patterns of repeats in different streptococcal GTFs vary, but no correlation has been made between these repeats and enzyme function. The repeats encoded by *S. gordonii* *gtfG* have been classified (42) by their similarity to the ca. 33-amino-acid "A" and ca. 20-amino acid "C" consensus sequences (6, 27). The first A repeat sequence of *S. gordonii* GTF has been designated A' and occurs at amino acid residue 1173.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Carboxyl-terminal repeat pattern	Source or reference
Bacteria			
<i>S. gordonii</i>			
CH1	Parental Challis strain	A'-A1-C1-A2-C2-A3-C3-A4-C4-A5-C5	36
AMS12	CH1 with 1.7-kb <i>Hind</i> III fragment of <i>gtfG</i> replaced with a <i>lacZ/erm^r</i> insertion		45
CH1(pAMS57)	<i>rgg</i> in pVA749; Em ^r		35
CH107	Spontaneous Spp ⁻ mutant with 585-bp <i>gtfG</i> deletion	A'-A1-C1-A2/5-C5	This study
CH107(pAMS57)	<i>rgg</i> in pVA749; Em ^r		This study
CH2RPE	GTF terminates 14 amino acids before the parental GTF carboxyl terminus; pVA891 insert, Em ^r	A'-A1-C1-A2-C2-A3-C3-A4-C4-A5-C5	This study
CH4RPE	GTF terminates 14 amino acids before the parental GTF carboxyl terminus and has two additional A-C repeats; pVA891 insert, Em ^r	A'-A1-C1-A2-C2-A3-C3-A4-C4/2-A3-C3-A4-C4-A5-C5	This study
<i>S. mutans</i>			
3209			J. J. Ferretti
ATCC 25175			J. J. Ferretti
Ingbritt			J. J. Ferretti
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>		BRL
Plasmids			
pVA749	Streptococcal cloning vector; Em ^r		21
pAMS40	9.2-kb CH1 insert containing <i>rgg</i> and <i>gtfG</i> and their flanking regions in pVA749		41
pAMS57	Subclone of pAMS40 that carries <i>rgg</i> in pVA749; Em ^r		35
pVA891	<i>E. coli</i> vector; Em ^r		20
pBluescript KS ⁺	<i>E. coli</i> vector; Ap ^r		Stratagene
pAM6207	700-bp CH1 <i>gtfG</i> repeat region in pBluescript KS ⁺		This study
pAM6206	700-bp insert from pAM6207 in pVA891		This study
pAMS21	3.4-kb <i>Hind</i> III fragment of pAMS40 in pBluescript KS ⁺		42
pAM6201	1.2-kb <i>Hind</i> III- <i>Xba</i> I subclone of pAMS21 in pBluescript KS ⁺		42

This is followed at amino acid 1237 by five tandem A and C repeats designated A1 and C1 through A5 and C5. Thus, the pattern of repeats in the enzyme encoded by *gtfG* is A-A-C-A-C-A-C-A-C-A-C. Although no correlation has been made between patterns of repeats and enzyme function, deletion studies of cloned GTF-I enzymes from three strains of mutans streptococci (1, 6, 17), which all have the same A-A-C-A-C-A-C-A-C-A-C repeat pattern as that of the *S. gordonii* GTF, suggest that a minimum of two A repeats are required for glucan binding and enzyme function. In contrast, the *S. mutans* GTF-S enzyme (15), which has five repeats similar to the A consensus repeat but no C consensus repeats, appears to require four A repeats for efficient glucan binding (19). To gain further insights into the role of the carboxyl-terminal repeats in GTF activity, the studies reported here describe *S. gordonii* strains with specific changes in the carboxyl terminus of *gtfG* and examine the effects of these changes on GTF expression and activity.

MATERIALS AND METHODS

Bacteria and plasmids. All strains were stored at -70°C in 50% glycerol. The bacterial strains and plasmids used in this study are shown in Table 1.

Media and chemicals. *S. gordonii* strains were grown in Todd-Hewitt (TH; Difco Laboratories, Detroit, Mich.) or defined FMC (37) medium and incubated in a candle jar or anaerobic chamber (Coy Industries, Jackson, Mich.) at 36°C. The sucrose-promoted colony phenotype (Spp) was determined to be positive or negative by a hard or soft colony phenotype, respectively (36), on 3% sucrose-

TH agar plates incubated for 48 h in 5% CO₂. *S. gordonii* strains carrying pAMS57 or transformant strains with integrated erythromycin resistance determinants were grown with 5 μ g of erythromycin per ml.

Escherichia coli DH5 α strains were grown in Luria-Bertani (LB) medium (2). Selection of strains with cloned inserts in pBluescript was done on agar plates with 100 μ g of ampicillin per ml, 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and 12 mM 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Selection of strains carrying cloned inserts in pVA891 was done with agar plates with 200 μ g of erythromycin per ml. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL; Gaithersburg, Md.), and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated.

DNA isolation and manipulations. Chromosomal and plasmid DNAs from *S. gordonii* were prepared as described previously (35). Plasmid DNA from *E. coli* was prepared with Qiagen purification columns (Qiagen, Inc., Chatsworth, Calif.) as described in the manufacturer's directions. In some cases, subcloned DNA fragments were eluted from agarose gels with Qiaex beads (Qiagen). DNA subclones or PCR fragments were digested with appropriate restriction enzymes, ligated with T4 DNA ligase into convenient restriction sites of the vector, and transformed into *E. coli* DH5 α by the CaCl₂ method (2). *S. gordonii* cells were transformed as described previously (35).

PCR. Double-stranded PCR products were obtained with a GeneAmp PCR kit in a model 480 DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) with AmpliTaq enzyme according to the manufacturer's directions as described previously (42). Custom oligonucleotide primers were synthesized at the DNA Core Facility (University of Michigan, Ann Arbor, Mich.). PCR products were cleaned with a Qiaex PCR purification kit (Qiagen).

Construction of subclones for nucleotide sequence determination of chromosomal DNA. To examine the nucleotide sequence of strain CH107, chromosomal DNA was used as template for PCR with the oligonucleotide primers listed in Fig. 1. Primers were designed to amplify areas of interest in *rgg*, *gtfG*, and their flanking regions. Primers had either engineered 5' restriction sites or else were designed to anneal immediately 5' to a convenient restriction site so that diges-

FIG. 1. Map of 9.2-kb insert of plasmid pAMS40 which consists of strain CH1 *Hind*III (H) fragments of 4.0, 0.1, 1.6, and 3.4 kb cloned into the streptococcal vector pVA749. Thick arrows above the map designate the positions of the *rgg* and *gtfG* ORFs. The inverted repeats which precede the *rgg* and *gtfG* ORFs as well as the inverted repeat downstream of *gtfG* are designated by inverted arrows. The positions of the *gtfG* signal sequence and putative catalytic active site region as well as the six direct repeats in the region encoding the carboxyl terminus are marked. Beneath the map are shown the relative positions of PCR-generated subclones used for determining the CH107 nucleotide sequence. Primers used to generate these fragments had an engineered flanking *Bam*HI or *Hind*III site (underlined) or else included, or were immediately upstream from, a convenient restriction site for cloning the double-stranded PCR product into the *Bam*HI (B), *Hind*III (H), or *Xba*I (X) sites of pBluescript KS⁺. In some cases, partial *Hind*III digestion of the PCR fragments was necessary for cloning. Areas in the parental carboxyl-terminal region that were not present in the strain CH107 subclones are indicated by dots.

tion of the fragment with appropriate restriction enzymes would allow ligation into the multiple-cloning region of pBluescript KS⁺.

Subclones for determining the nucleotide sequence of the region of *gtfG* encoding the carboxyl terminus and to confirm the site of the integrated pVA891 in strains CH2RPE and CH4RPE were constructed similarly by PCR with a chromosomal template. The oligonucleotide primers 5'CTTCAATGGTACCAATATCTTAGGTCG3', designed to anneal 122 bp upstream from the *Hind*III site immediately upstream from the direct repeat region (see map of *gtfG* in Fig. 1), and 5'AGGTGCTGACTTTCAACTGC3', designed to anneal 54 bp downstream from the *Hind*III site in the integrated pVA891, were used. The resulting PCR products from strains CH2RPE and CH4RPE were digested with *Hind*III and cloned into pBluescript KS⁺.

In each strain, each PCR gave only one product. To guard against mistakes due to misincorporation of nucleotides during PCR, three independent products from each strain were sequenced.

Nucleotide sequence determination and analysis. Double-stranded DNA sequencing was done by a modification (42) of the dideoxy chain termination sequencing method (29) with the Sequenase Version 1.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with M13 forward and reverse primers for the pBluescript cloning vector and custom oligonucleotide primers as necessary. To determine the sequence of direct repeat regions that did not have unique sites for custom primers, a cycle sequencing method which yielded ca. 750 readable bases per reaction was used at the University of Michigan DNA Sequencing Facility. Templates were sequenced with a PRISM-Ready Reaction Dye Deoxy Terminator Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.) by using AmpliTaq enzyme as described in the manufacturer's directions. Products were labelled for 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Unincorporated dideoxynucleoside triphosphates were removed, and samples were concentrated, reconstituted in loading buffer, denatured, and run on 4 or 6% polyacrylamide gels 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, Inc., New Haven, Conn.) and compared and analyzed with the MacVector software program (International Biotechnologies).

Southern hybridization analyses. DNA was digested with appropriate restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to a Hybond-N (Amersham) membrane by 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, Ind.) as described in the manufacturer's directions.

Determination of extracellular GTF activity. Relative extracellular GTF activity of each strain was determined by two methods. Strains were grown in FMC medium to the same mid- to late-log stage (optical density at 520 nm, 1.6), and

equal volumes of cell-free supernatant were run on sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE) activity gels, as described previously (36). Relative GTF activity was determined by quantitation of the intensity of stained glucan bands in at least four independent GTF activity gels by using an image acquisition and software system (Ambis Inc., San Diego, Calif.). GTF activity was expressed as a percentage of the activity of the parental strain CH1 (100%) as described previously (35).

Extracellular GTF activity of each strain was also determined by measuring the ability of equal volumes of FMC culture supernatants to incorporate [¹⁴C]-glucose/sucrose (NEN Dupont, Boston, Mass.) into methanol-precipitated total glucan polymer (25). Strain AMS12 was included as a negative control, and the incorporated radiolabelled counts from this strain (which were always less than 1% of that of strain CH1) were subtracted from the values for each strain. Tests were done in triplicate and repeated three times. Values are expressed as a percentage of the radiolabelled counts incorporated into glucan by strain CH1 (100%) in the same test.

Western blot (immunoblot) analysis of GTF antibody-reactive proteins. *S. gordonii* cultures were grown to the same mid- to late-log growth phase (optical density at 520 nm, 1.7). Bacteria were removed by centrifugation at 1,000 × *g*. Cell-free supernatants were concentrated 10-fold in Centricon-50 concentrators (50-kDa cutoff; Amicon, Inc. Beverly, Mass.), and equal volumes were loaded onto SDS-7.5% acrylamide gels (18). After electrophoresis for 12 to 16 h at 8 mA per gel, proteins were transferred with a transblot cell (Bio-Rad Laboratories, Hercules, Calif.) at 100 V onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer (pH 11.0). Membranes were blocked with 5% milk in Tris-buffered saline (25 mM Tris, 0.5 M NaCl [pH 7.4]). Proteins were detected with polyclonal anti-GTF-I rabbit antiserum raised against the product of *S. mutans* GS5 *gtfB* (a gift of H. K. Kuramitsu). On the basis of amino acid sequences deduced from the nucleotide sequences (42), the GTF-I protein is 51.5% identical to *S. gordonii* CH1 GTF as determined by the Genetics Computer Group (Wisconsin) Gap program for protein similarity (13) and could detect different quantities of *S. gordonii* strain CH1 GTF protein in culture supernatants (40). This antibody was diluted 1:200 in a solution of 3% milk-0.03% Tween 20 in Tris-buffered saline and incubated overnight at 4°C. Primary antibody binding was detected with goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (BRL) and 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium substrate (Sigma). Comparative quantitation was done by scanning three independent blots with an Ambis image acquisition and software system. Values for each strain are expressed as a percentage of strain CH1 antibody-reactive protein (designated as 100%).

Preparation of partially purified GTF. Cultures were grown anaerobically in FMC medium with 1 mM phenylmethylsulfonyl fluoride until the late log phase (optical density at 520 nm, 1.9) and spun at 1,000 × *g* to remove cells. The

supernatants were concentrated 20-fold in Centricon-50 concentrators. A comparison of silver-stained and GTF activity SDS-PAGE gels indicated that GTFs were predominant proteins in these preparations. Total protein concentrations were determined by the method of Markwell et al. (22).

Glucan binding assay. The glucan binding ability of the various GTFs was examined by a modification of the assay of Lis et al. (19). In this assay system, glucan binding ability is determined as the ability of partially purified GTFs from defined medium culture supernatants (prepared as described above) fixed to microtiter plates to bind to biotin-dextran. Thirty micrograms of protein containing partially purified GTFs from each strain was suspended in carbonate coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 [pH 9.7]) in microtiter plate wells (Dynatech Laboratories, Inc., Chantilly, Va.). After overnight incubation at 4°C, the wells were washed with a microtiter plate washer (Elcatech, Inc., Winston Salem, N.C.), and 200 μl of blocking buffer (0.5% [wt/vol] bovine serum albumin in 10 mM sodium acetate [pH 6.0]) was added to the wells and incubated at 37°C for 1 h. The wells were washed again, and then increasing concentrations of biotin-dextran (70 kDa; Sigma) were added to the wells and the wells were incubated at room temperature for 10 min. The wells were washed again, 100 μl of a 1:1,000 dilution of alkaline phosphatase-streptavidin (Zymed Laboratories Inc., South San Francisco, Calif.) was added to the wells, and the wells were incubated for 5 min. After a final wash, 100 μl of a 1:1,000 dilution of Sigma 104 phosphatase substrate in buffer (28 mM NaHCO_3 , 22 mM Na_2CO_3 , 5 mM MgCl_2 [pH 9.7]) was added, and the change in A_{405} was measured with a Perkin-Elmer Lambda Automated Microplate Reader.

In vitro glucan synthesis. One milliliter of partially purified extracellular GTF from each *S. gordonii* strain was added to 49 ml of filter-sterilized substrate solution consisting of 5% (wt/vol) sucrose and 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 three volumes of 95% ethanol at -20°C and collected by centrifugation at $16,000 \times g$. The glucans were washed with water and reprecipitated three times. The final washed glucan products were dried in a Speedvac concentrator (Savant Instruments Inc., Farmingdale, N.Y.).

^{13}C NMR analysis of glucans. Glucans produced in vitro by partially purified enzymes from each *S. gordonii* strain were dissolved in dimethyl sulfoxide until saturated and examined at the University of Michigan NMR Core Facility in a GE Omega 500 spectrometer. ^{13}C nuclear magnetic resonance (NMR) spectra were obtained at 124 MHz in a 10-mm tube with a probe temperature of 75°C. The spectra consisted of 16,000 datum points over a spectral width of 26,315 Hz for 60,000 scans. Relaxation time was 2.0 s. Digital resolution was 3.2 Hz. The internal reference was dimethyl sulfoxide at 40 ppm. Peak assignments for carbons of glucose moieties in $\alpha 1,6$ and $\alpha 1,3$ linkages were based on published values (5, 30). Relative proportions of carbons in each linkage were determined by calculating the area under each carbon peak with GE Omega version 6.0.2 software and assigning the total area under all carbon peaks the value of 1.00. Proportions of carbons in each linkage within glucans were determined by the relative area under each peak (31).

RESULTS

Characterization of strain CH107. A spontaneous mutant was identified by its Spp^- colony morphology and designated strain CH107. This strain differed from Spp^- GTF reversible phase-variant strains in several ways. Although *S. gordonii* Spp^- phase-variant strains are also soft on sucrose agar plates, they revert to Spp^+ at frequencies of 10^{-5} to 10^{-3} (36, 39). No reversion of strain CH107 to Spp^+ was detected (frequency, $<5 \times 10^7$). Activity gels showed that this strain had not only a decreased level of GTF activity but also a lower apparent molecular weight of the native enzyme of ca. 153,000 compared with the ca. 174,000-molecular-weight parental native GTF (Fig. 2A, lanes 1 and 2).

Western blot analyses showed an additional way in which strain CH107 differed from Spp^- phase-variant strains. Whereas Spp^- phase variants have ca. fivefold-decreased levels of extracellular GTF protein (40), strain CH107 culture supernatants had levels of GTF antibody-reactive protein similar to that of the parent (Fig. 2B, lanes 3 and 6). Transformation of strain CH107 with pAMS57, which carries *rgg*, increased GTF activity about fivefold (Fig. 2A, lane 3), similar to the results seen when strain CH1 is transformed with pAMS57 (35). Furthermore, Western blots confirmed that *rgg* increased the amount of extracellular protein in strains CH1 and CH107 to similar extents (Fig. 2B, lanes 2 and 7). However, unlike the results seen with reversible Spp^- phase-variant strains (16, 35),

FIG. 2. GTF activity and GTF-I antibody-reactive protein in FMC culture supernatants. (A) Activity gels. Cultures were grown to the same mid- to late-log phase (optical density at 520 nm, 1.7) in FMC medium. Cell-free supernatants were run on an SDS-7.5% PAGE gel and incubated in 3% sucrose with 0.5% Triton X-100, and the resulting glucans were stained to determine GTF activity (36). Beneath the native protein band are lower-molecular-weight bands which are thought to be due to the degradation of the native enzyme by endogenous proteases (12). Lanes: 1, the parental strain CH1; 2, strain CH107; 3, strain CH107(pAMS57); 4, the GTF-negative control strain AMS12; 5, strain CH4RPE; 6, strain CH2RPE. (B) Western blots. Aliquots from similarly prepared culture supernatants were run on an SDS-7.5% PAGE gel, transferred to polyvinylidene difluoride membranes and probed with antiserum raised against *S. mutans* GTF-I to detect antibody-reactive proteins as described in Materials and Methods. Lanes: 1, strain AMS12; 2, strain CH1(pAMS57); 3, strain CH1; 4, strain CH2RPE; 5, strain CH4RPE; 6, strain CH107; 7, strain CH107 (pAMS57). Protein size standards (BRL) are indicated on the right.

providing *rgg* in *trans* did not confer an Spp^+ colony morphology on the Spp^- strain CH107.

To determine the basis of this decreased size of the strain CH107 GTF, Southern hybridization analysis was done. Chromosomal DNA from strains CH1 and CH107 was digested with *Hind*III and probed with pAMS40 (41), which carries a 9.2-kb insert of strain CH1 *rgg*, *gtfG*, and their flanking regions (see map in Fig. 1). As expected, in the parental CH1 strain, the probe hybridized with four *Hind*III fragments: (i) a ca. 4-kb fragment that contains *rgg* and the first ca. 1.6 kb of *gtfG*, (ii) a ca. 100-bp fragment, (iii) a ca. 1.7-kb fragment that contains the middle portion of *gtfG*, and (iv) a ca. 3.4-kb band that contains the region encoding the carboxyl-terminal repeats of *gtfG*, the stop codon, and the downstream region which includes a region of dyad symmetry thought to be involved in transcriptional termination (42). The 4-kb, 1.6-kb, and 100-bp fragments each appeared to be the same size in strains CH1 and CH107 (Fig. 3). However, rather than hybridizing to the 3.4-kb fragment seen in CH1 and pAMS40, the probe hybridized to a 2.8-kb fragment in strain CH107, suggesting that a deletion may have occurred in the region encoding the carboxyl terminus of *gtfG* in strain CH107.

Nucleotide sequence analysis of strain CH107. To examine the possibility that such a deletion had occurred, subclones of

FIG. 3. Southern hybridization analysis of strain CH107. Chromosomal DNA of strains CH1 and CH107 as well as pAMS40 was digested with *Hind*III, electrophoresed on 0.7% agarose, transferred to a Hybond-N membrane, and hybridized under stringent conditions to pAMS40 DNA labelled with digoxigenin-dUTP. Fragments that hybridized to the probe were detected by chemiluminescence. DNA size standards (BRL) are indicated on the left. The pAMS40 control lane shows a ca. 5.3-kb band that corresponds to the pVA749 vector, and bands corresponding to the ca. 4.0-, 1.7-, and 3.4-kb fragments are shown in the pAMS40 insert map in Fig. 1. The 100-bp *Hind*III fragment in the middle of *gtfG*, which was first detected by nucleotide sequence analysis (42) and requires overloading of the gel for visualization, is not shown.

the strain CH107 *gtfG* region encoding the carboxyl terminus were generated with the second and third pairs of primers shown in Fig. 1. The nucleotide sequence was determined and confirmed that a 585-bp deletion occurred in the direct repeats of the strain CH107 *gtfG* (Fig. 4). This deletion is probably due to spontaneous deletion and recombination between the internal portion of the repeat region containing the A-C repeats that are most homologous to each other (42). A portion of the A2 repeat appears to have recombined in frame with the A5 repeat. The upstream A' repeat and the downstream C5 repeat, which are the least-conserved repeats, are maintained intact in strain CH107. Thus, the carboxyl-terminal region of strain CH107 GTF contains an upstream A' repeat and two A-C repeats; compared with the parental pattern, the strain CH107 pattern is A'-A1-C1-A2/5-C5 (Table 1). The region downstream of the deleted region, which included the TAA stop codon and downstream region of dyad symmetry, was identical to that of the parent (Fig. 4).

To confirm that the decreased GTF activity seen in strain CH107 was not due to mutations in *rgg* or changes in other regions of *gtfG* that were not detected by Southern hybridization analysis, additional PCR-generated subclones were made (Fig. 1) and their nucleotide sequences were determined to be identical to that of strain CH1. Thus, the *gtfG* open reading frame (ORF) of strain CH107 consists of 4,149 bp encoding a protein of 1,382 amino acids. On the basis of the nucleotide sequence data, the deduced molecular weight of the preprocessed protein is 155,941; the processed protein has a deduced molecular weight of 152,025 with an estimated pI of 5.14.

Construction of mutants with specific changes in the carboxyl terminus of *gtfG*. The 22-bp sequence at the start of the

CH107 deletion occurred three times at ca. 200-bp intervals in the parental *gtfG* (Fig. 4). These ca. 200-bp regions were 87.7 to 91.3% identical to each other, and their deletion from the strain CH107 *gtfG* showed their potential for homologous recombination with each other. On the basis of these findings, an approach was designed to construct mutants with different numbers of direct repeats in *gtfG* by transforming strain CH1 with a suicide vector carrying these three 200-bp sequences. Theoretically, recombination could occur between any of the 200-bp segments on the plasmid and any of the 200-bp segments of the chromosomal *gtfG*, and therefore strains with the integrated plasmid could have *gtfG* ORFs of various sizes. These strains, with different numbers of direct repeats, could provide insights into the relationship between glucan binding and enzyme activity.

To construct the repeat fragment, PCR primers were designed to minimize any conserved flanking DNA that might favor recombination between corresponding 200-bp segments of the plasmid and chromosomal *gtfG* yet maximize the size of the resulting *gtfG* ORF when plasmid integration occurred. The forward primer 5'-GGATCCCAAGTCAAGGGTCAAGTAGTGA3' was designed with an engineered *Bam*HI (GGA TCC) site and the first 22 bp of the region deleted from strain CH107. The reverse primer, 5'-AAGCTTACATCCAAGTTC CGTCTTCTAG3', was designed to anneal downstream of the fifth repeat that was similar to the C consensus (C5 [Fig. 4]); this included a flanking engineered *Hind*III (AAGCTT) site to facilitate cloning and an engineered stop site (TAA) to avoid translation of vector-encoded amino acids into the carboxyl terminus of the resulting GTF. These primers were used in PCR with pAMS21 (42), a subclone of pAMS40 that carries the carboxyl-terminal repeat region of *gtfG*, as template DNA. As expected, the reaction produced three fragments that differed in size by ca. 200 bp. The largest fragment (ca. 700 bp) was eluted from a 1.5% agarose gel, cloned into pBluescript KS⁺, and designated pAM6207. After nucleotide sequence analysis to confirm that there had been no misincorporations during PCR, the 700-bp fragment from pAM6207 (which had the nucleotide sequence that is enclosed in brackets in Fig. 4) was cloned into the *Bam*HI and *Hind*III sites of the suicide vector pVA891 and designated pAM6206.

Characterization of the carboxyl-terminal insertional mutants. Strain CH1 cells were transformed with pAM6206, and 40 erythromycin-resistant transformants were selected randomly for analysis. These colonies were all Spp⁺ on sucrose agar plates but appeared slightly less glossy than the parental colony phenotype. Culture supernatants were examined on GTF activity gels, and 39 of these strains produced GTFs that had a just slightly lower molecular mass than the 174-kDa parental GTF. Southern hybridization analysis of these strains suggested that when the plasmid carrying the repeats integrated into the chromosome, recombination occurred between the corresponding 200-bp similar sequences on the plasmid and chromosome (Fig. 5A). If these strains arose in this manner, then digestion of their chromosomal DNA with *Hind*III would result in a 1,275-bp fragment that contained DNA from nucleotide 3416 of *gtfG* through the engineered stop site. It would also yield a ca. 8.9-kb fragment consisting of the ca. 6.1-kb integrated pVA891 vector, 700 bp of repeat DNA, the remaining 42 bp of the *gtfG* ORF, and the ca. 2.1 kb of DNA downstream of the *gtfG* ORF (see map of pAMS40 in Fig. 1). This indeed occurred in the 39 strains examined. As shown in the representative strain chosen for further analysis and designated CH2RPE (Fig. 6), when the *Hind*III-digested chromosomal DNA was probed with the ca. 1.2-kb insert of pAM6201 (42), two fragments of ca. 1.25 and 8.9 kb hybridized

FIG. 4. Nucleotide and deduced amino acid sequences of the carboxyl-terminal region of the parental strain CH1 *gtfG*. Numbers of the first nucleotide and amino acid in each line are shown in the left margin and are based on designating the first nucleotide and amino acid of the ORF 1. The complete nucleotide sequence of *gtfG* is deposited in GenBank under accession number U12643 (42). The *HindIII* site just upstream from the direct repeat region is indicated by an overline (see *gtfG* map [Fig. 1]). The *XbaI* site directly downstream from the direct repeats is similarly marked. The six regions similar to the A consensus amino acid sequence are designated by bold characters and designated A' and A1 through A5. The five regions similar to the C consensus sequence are designated by italics and labelled C1 through C5. The region of dyad symmetry (nucleotides 4787 through 4831) which may function in transcription termination is designated by inverted arrows. The 585 nucleotides deleted in strain CH107 (nucleotides 3994 through 4578) are labelled and designated with arrows. The 699-bp region amplified by PCR, cloned into pAM6206, and then used to construct strains CH2RPE and CH4RPE is set off by brackets. The three identical sequences that annealed to the forward primer used for PCR of this region are underlined. The sequence complementary to the reverse PCR primer, which contained an engineered TAA translational stop site (in parentheses above nucleotide 4692), is designated with a dotted line. Strain CH2RPE appears to have arisen through an in-frame homologous recombination between the plasmid and chromosome (see Fig. 5A), and therefore its nucleotide sequence is identical to the parental sequence until the engineered stop site. Nucleotide sequence analysis of strain CH4RPE indicated that it arose as a result of a recombination between the 21-bp sequence (nucleotides 4450 through 4470) designated by parentheses in the chromosome in the C4 repeat with the first homologous repeat in pAM6206 (corresponding to chromosomal nucleotides 4060 through 4080 in the C2 repeat). This gave rise to a *gtfG* ORF with an additional 390 bp encoding an additional two tandem A-C repeats. Like that of similarly constructed strain CH2RPE, the ORF of strain CH4RPE has an engineered TAA translational stop codon and ends at the bracketed region, which is then followed by the integrated pVA891 vector.

to the probe. A ca. 8.9-kb fragment hybridized to the pVA891 probe.

Although 39 of the selected transformants showed a GTF activity band of ca. 174 kDa, one strain, designated CH4RPE, produced a native GTF of ca. 190 kDa (Fig. 2A, lane 5). Southern hybridization analysis of *HindIII*-digested DNA of this strain showed that the 1.2-kb insert of pAM6201 hybridized to a ca. 1.6-kb fragment and a ca. 8.5-kb fragment. The pVA891 probe hybridized to a ca. 8.5-kb fragment (Fig. 6). These results suggest that strain CH4RPE arose by a recombination between pAM6206 and the chromosomal *gtfG* that increased the size of the *gtfG* ORF by ca. 400 bp and decreased the size of the downstream *HindIII* fragment carrying pVA891

by ca. 400 bp. This result is consistent with a recombination of the third 200-bp segment of the chromosomal *gtfG* and the first 200-bp segment in pAM6206 (Fig. 5B).

HindIII-digested DNA from strains CH1, CH2RPE, and CH4RPE all had fragments of the same size hybridize to the 4.0- and 1.6-kb *HindIII* fragments of pAMS40 (see map in Fig. 1; data not shown) suggesting that, as expected, the only change in the *gtfG* ORFs of strains CH2RPE and CH4RPE occurred in the region encoding the carboxyl terminus.

Nucleotide sequence analysis of strains CH2RPE and CH4RPE. To confirm the site of integration of pAM6206 and determine the nucleotide sequences of the *gtfG* genes in strains CH2RPE and CH4RPE, the carboxyl-terminal regions were

FIG. 5. Construction of strains CH2RPE and CH4RPE. Potential recombinations between the three 200-bp segments within the ca. 700-bp insert of pAM6206 and the chromosomal *gtfG* that could result in strains with changes in the carboxyl terminus of *gtfG* are shown. (A) Homologous recombination between corresponding 200-bp regions in the repeat fragment of pAM6206 and the chromosomal *gtfG* as occurred in the representative strain CH2RPE; (B) homologous recombination between the first 200-bp region of the repeat fragment in pAM6206 with the third 200-bp region of *gtfG* on the chromosome as occurred in strain CH4RPE. Although recombination between plasmid and chromosomal 200-bp regions could potentially have occurred in a number of other ways, no other recombination and integration patterns were detected among the transformants screened.

amplified by PCR and cloned into pBluescript KS⁺ for sequencing. PCR products which included the region from nucleotide 3294 of *gtfG* and 52 bp downstream from the *Hind*III site in the integrated pVA891 were ca. 1.4 kb and ca. 1.8 kb for strains CH2RPE and CH4RPE, respectively, as predicted from the Southern hybridization analyses. Nucleotide sequence analysis of strain CH2RPE confirmed that the recombination between pAM6206 and the chromosome was in frame and the sequence was identical to that of the parent up to the engineered stop site. Therefore, the *gtfG* of strain CH2RPE differs from that of the parent by only the absence of 42 nucleotides encoding the 14 carboxyl-terminal amino acids. The complete CH2RPE *gtfG* ORF of 4,692 bp encodes a processed protein with a deduced molecular weight of 172,122 and a pI of 5.06.

Nucleotide sequence analysis of strain CH4RPE confirmed that the *gtfG* of this strain probably occurred by in-frame recombination between the third 200-bp region in the chromosome with the first 200-bp region of pAM6206 (Fig. 5B). The sequence of the *gtfG* ORF was identical to that of the parent up to nucleotide 4470 (Fig. 4). Thereafter, the sequence was identical to the sequence from nucleotide 4081 through the engineered TAA stop site, resulting in a 5,082-bp ORF. Thus, recombination appears to have occurred somewhere within the

21-bp sequence GAGATGGGCGTCAACAAATTT (region enclosed by parentheses at the site of chromosomal recombination in Fig. 4) which occurs at nucleotide positions 4450 to 4470 and also at positions 4060 to 4080. The recombinant CH4RPE *gtfG*, therefore, encodes two additional repeats similar to the A and C consensus repeat sequences. Like the GTF of CH2RPE, the GTF of CH4RPE is missing the 14 carboxyl-terminal amino acids. The deduced amino acid sequence of CH4RPE *gtfG* suggests that the molecular weight of the processed GTF is 186,579 and the pI is 5.07.

Relative extracellular GTF activity. Like the parental strain CH1, strains CH107, CH2RPE, and CH4RPE had two major forms of active GTF that differed by ca. 18 kDa (Fig. 2A). The deletion of three A-C repeats in strain CH107 clearly decreased the level of extracellular GTF activity to less than 15% of that of the parental strain as determined by both GTF activity gels and [¹⁴C-glucose]sucrose incorporation (Fig. 7). Strain CH2RPE had slightly less GTF activity than strain CH1 did; strain CH4RPE, which had two additional A-C repeats, had a slight increase in GTF activity compared with strain CH2RPE. However, the differences between pairs of activity levels of strains CH1, CH2RPE, and CH4RPE were not sta-

FIG. 6. Southern hybridization analysis of *Hind*III-digested chromosomal DNAs from strains CH1, CH2RPE, and CH4RPE. Digoxigenin-dUTP-labelled probes were hybridized to the membranes under stringent conditions and detected by chemiluminescence. (A) DNA was probed with a 1.2-kb *Hind*III-*Xba*I fragment of pAM6201 (42) which contains the region encoding the carboxyl terminus of *gtfG* (see map, Fig. 1). (B) DNA was probed with pVA891. DNA size markers are indicated on the right. All three strains had the same-size fragment hybridize to the 4.0- and 1.6-kb *Hind*III fragments of the pAMS40 probe (data not shown), suggesting that the only change in the *gtfG* ORFs of strains CH2RPE and CH4RPE occurred in the region encoding the carboxyl terminus.

tistically significant ($P > 0.07$), perhaps in part because of the inherent variability of both assay methods.

Glucan binding abilities of the GTFs. The ability of partially purified GTF preparations fixed to microtiter plates to bind α 1,6-linked biotin-dextran showed a linear increase with increasing dextran concentrations for strains CH1, CH2RPE, and CH4RPE (Fig. 8A). Strain CH2RPE showed consistently less glucan binding ability than strain CH1, even though their A-C repeat patterns were identical. Strain CH4RPE, which had two additional A-C repeats, showed increased glucan binding ability to a level greater than that of strain CH1. However, despite the fact the strain CH107 had GTF activity and, therefore, must have some glucan binding ability, it was not detected in this test system. Strain CH107 showed no more glucan binding ability than the negative control strain AMS12 or blank wells with buffer alone; no differences were seen when CH107 protein concentrations of up to 90 μ g were bound to the microtiter plate wells or a concentration of 500 μ g of dextran per ml was used (data not shown).

Similarly prepared supernatant extracts of representative *S. mutans* strains (Fig. 8B) which have multiple GTF enzymes (27) as well as additional glucan-binding proteins (3, 33) were also examined. Equal amounts of protein from *S. gordonii*

FIG. 7. Relative extracellular GTF activity for each strain expressed as a percentage of GTF activity of strain CH1 (100%). Error bars indicate standard deviations. ■, GTF activity as determined by [14 C]glucose sucrose incorporation into methanol-precipitated glucans (tests were done in triplicate and repeated three times); ▨, GTF activity as determined by scanning the intensities of glucan bands on SDS-PAGE GTF activity gels of culture supernatants from each strain (at least four independent GTF activity gels were examined).

strain CH1 showed glucan binding ability similar to those of the mutans streptococci.

Western blot analyses of GTF antibody-reactive protein. To determine whether the levels of GTF activity corresponded to the amount of extracellular GTF protein present, Western blotting was done. Although the primary antibody used in these studies was raised against the product of the *S. mutans gtfB*, control blots confirmed that it was sufficiently cross-reactive to detect the *S. gordonii* GTF (40). GTF activity gels and Western blots made with aliquots of the same preparations confirmed that the sizes of the bands with glucan activity corresponded to the sizes of the antibody-reactive bands. Cultures grown in FMC medium showed more lower-molecular-weight forms of the enzyme than cultures grown in TH broth did, presumably as a result of the ability of complex proteins present in TH medium to more efficiently protect the GTF from endogenous proteases (11). However, scanning of Western blots (Fig. 2B) indicated that similar amounts of antibody-reactive protein were present in the culture supernatants of strains CH1, CH2RPE, CH4RPE, and CH107 (values ranged from 86 to 108% of the parental strain CH1 protein with standard deviations of $\leq 12\%$), suggesting that in *S. gordonii*, differences in the number of A and C consensus carboxyl-terminal repeats did not significantly affect the amount of extracellular GTF antibody-reactive protein. Neither did the deletion of the 14 terminal amino acids of the GTF in strain CH2RPE appear to significantly affect the level of extracellular GTF activity or protein as determined by these assays.

NMR analysis of glucans synthesized in vitro. The structures of the glucans produced by each strain were examined. The 13 C NMR spectrum for the parental strain CH1 (Fig. 9A) was similar to previously published spectra (14) and showed carbon peaks representing glucose moieties in both α 1,6 and α 1,3 glucosidic linkages. Spectra from strains CH2RPE and CH4RPE (Fig. 9C and D, respectively) appeared similar to that of the parent, suggesting that the glucan products of these strains were similar. Glucans produced by strain CH107 were difficult to solubilize in dimethyl sulfoxide for NMR analysis and had a less-than-optimum signal-to-noise ratio. However, the spectrum (Fig. 9B) was sufficient for analysis and clearly

FIG. 8. Ability of partially purified GTFs fixed to microtiter plates to bind various concentrations of biotin-dextran as determined by change in color reaction at 405 nm. (A) Representative test for the *S. gordonii* strains; (B) relative percentage of binding ($\pm$ standard deviation, $n = 3$) compared with that of strain CH1 (100%) of 1 μ g of biotin-dextran per ml, a concentration at which the titration curve was linear for each strain. Partially purified GTF supernatant samples for the *S. gordonii* strains and the representative *S. mutans* strains 3209, ATCC 25175, and Ingbritt were tested.

showed peaks at positions representing glucans with α 1,6 linkages (5). Absorbance peaks were not detected at positions for carbons in α 1,3 linkages. Even if peaks for α 1,3 linkages were present but obscured by the noise in the spectra, these peaks would not represent major components of the glucans produced by strain CH107. Thus, the glucans produced by strain CH107 had a significantly higher proportion of α 1,6 linkages than those produced by the other strains in this study.

DISCUSSION

The spontaneous *Spp*⁻ strain CH107 differed from *S. gordonii* reversible *Spp*⁻ phase-variant strains (36) in several ways. All examined *gtfG* genes of *Spp*⁻ phase-variant strains are identical to that of the parent, and accordingly, the encoded GTF enzymes of *Spp*⁻ phase variants have the same molecular weights (40). In the phase-variant strains, the basis of decreased GTF activity appears to relate, at least in part, to a quantitative difference in the amount of extracellular GTF protein compared with that of the *Spp*⁺ parent (40). In contrast, the basis of the decreased GTF activity of strain CH107 was not due to a decreased amount of extracellular GTF protein but rather to the structure of the enzyme itself. The internal deletion within the direct repeat region of the strain CH107 *gtfG* resulted in a protein with both decreased size and decreased activity. Furthermore, whereas the glucans produced

by the GTF enzymes of *Spp*⁻ phase-variant strains are similar to those of the parent (40), the glucans produced by strain CH107 differed significantly from those of the parent and had a significantly increased proportion of α 1,6 glucosidic linkages. Transformation of *Spp*⁻ phase variants with pAMS57, which carries *rgg* to increase the level of GTF activity, confers the *Spp*⁺ colony phenotype on these strains (16, 35). Although the transformant strain CH107(pAMS57) had a significant increase in GTF activity as expected, the colonies remained soft on sucrose agar plates. These results imply that a certain proportion of α 1,3 linkages in the glucans produced are necessary for a hard, *Spp*⁺ colony phenotype. Thus, the *Spp*⁻ colony morphology of strain CH107 is due to a qualitative rather than quantitative change in its GTF.

When comparing the relative activities of the different GTFs in this study, the proteolysis of the native GTF enzyme that occurs, even in the presence of protease inhibitors, and the resulting multiple forms of active enzyme (11) must be considered. The GTF proteins of strains CH1, CH107, CH2RPE, and CH4RPE all had major active forms of GTF that were ca. 18 kDa smaller than the native protein as well as lower-molecular-mass bands showing weaker GTF activity. Both GTF assays used in this study measure the activity of all active forms of GTF from the supernatants of each strain. Since the strain CH4RPE GTF has two additional A-C repeats, it is possible that as proteolysis occurs, the larger GTF of strain CH4RPE could have more degraded forms that maintain activity, and therefore, more active bands would contribute to the total extracellular GTF activity of this strain. Although strain CH4RPE potentially had more active forms of GTF and showed more glucan binding ability in the microtiter plate assay, it did not have significantly more extracellular GTF activity than did the parent or strain CH2RPE, indicating that the additional A-C repeats did not significantly affect enzyme activity.

The glucan binding assay used in these studies requires the assumptions that the various GTFs being tested will attach to the microtiter plates with equal affinity and that these proteins can bind biotin-dextran as well as they can bind unbiotinylated dextran. It is interesting that these assays suggested that the *S. gordonii* GTF may be as efficient at binding α 1,6-linked glucans as some *S. mutans* GTFs are. The test was not able to detect glucan binding in strain CH107 even though this strain had GTF activity; however, the limited ability of this assay to detect very weak or inefficient glucan binding was also noted for deletion derivatives of *S. mutans* GTF-S (19). Nevertheless, the relative glucan binding abilities of the *S. gordonii* GTFs were directly related to the number of A-C repeats, with one notable exception. Strain CH2RPE, which had the same number of A-C repeats as the parent, was significantly less able to bind biotin-dextran. This could be due to conformational changes in the CH2RPE GTF. Deletion of 14 carboxyl-terminal amino acids, which included five positively charged arginine residues, one negatively charged aspartic acid residue, two uncharged hydrophobic residues, and six uncharged hydrophilic residues, could potentially affect GTF binding to the microtiter plate or binding of the biotin-dextran. Similarly, these putative conformational changes could affect enzyme activity. Indeed, the extracellular GTF activity of strain CH2RPE was approximately 10% less than that of strain CH1; however, within the limits of the assays used, this difference was not statistically significant.

The deletion of 14 amino acids from the carboxyl terminus and the resulting effects on protein conformation could also affect the ability of the different GTFs to be recognized by the polyclonal antibody used in the Western blot analyses. Simi-

FIG. 9. ¹³C NMR spectra of glucans produced in vitro from partially purified GTF enzymes from strains CH1 (A), CH107 (B), CH4RPE (C), and CH2RPE (D). Peaks were assigned for each carbon in glucose moieties in α1,6 and α1,3 glucosidic linkages and designated C1 through C6 and C'1 through C'6, respectively.

larly, since the carboxyl-terminal repeats of GTFs are thought to be immunogenic (34), it is possible that the number of repeats in the proteins being compared could affect the amount of antibody binding. Thus, strain CH4RPE GTF could have more antibody-reactive epitopes than that of the parent; strain CH107 GTF could have fewer. Despite the caution necessary in interpretation, the Western blot analyses suggest that strains CH1, CH107, CH2RPE, and CH4RPE all have similar amounts of extracellular GTF antibody-reactive protein.

The strains examined in this study have provided insights into the role of the direct repeats in *S. gordonii* GTF. Deletion of three A-C repeats in strain CH107 GTF significantly decreased the amount of glucan binding and enzyme activity although it did not decrease the amount of extracellular GTF protein. These findings are consistent with results from studies of nested deletions of *gtf* clones, which indicate that loss of one direct repeat significantly decreases the glucan binding and activity of *S. mutans* GTF-S (19) and that at least two A repeats are necessary for GTF-I enzymes of *S. mutans* (17), *Streptococcus downei* (6), and *Streptococcus sobrinus* (1) to produce glucan. Although no functional correlation has been made between repeat patterns and the type of glucan made by oral streptococcal GTFs, it is interesting that *S. gordonii* GTF has the same repeat pattern as the GTF-Is from these three strains. Although the parental *S. gordonii* GTF makes both soluble and

insoluble glucans, the loss of three repeats in strain CH107 resulted in a GTF that made apparently totally water-soluble glucans (data not shown) with primarily α1,6 glucosidic linkages. However, studies with hybrid *S. mutans* GTF enzymes (26) suggest that the repeat region alone does not determine the solubility of the product. Amino acid residue changes in several regions of *S. mutans* GTF have been found to affect the solubility of the glucans produced (32). The results of in vitro glucan synthesis in the present study also do not preclude the possibility that in vivo, bacterial or environmental factors could interact with the *S. gordonii* GTF and influence the nature of the glucan products. The studies described here only evaluated extracellular GTF. Although the relative amount of cell-associated GTF of *S. gordonii* strain Challis is low ($\leq 10\%$ of total culture GTF for strain CH1) (unpublished results) compared with the amount of extracellular enzyme (4), cell-associated GTF could play significant ecological roles and will be examined in future studies.

The spontaneously occurring *gtfG* of strain CH107 is consistent with the previous hypothesis that recombination can occur within the highly similar conserved direct repeats in the region encoding the carboxyl one-third of *S. gordonii* GTF (42). The potential also exists for intragenic recombination of direct repeats in *gtfG* that would result in a GTF of increased size. However, as seen in strain CH4RPE, the colony morphology of these strains could be very similar to that of the parent and

thus difficult to detect. The frequency of such an event would be expected to be low, and no spontaneously occurring strains with increased numbers of GTF direct repeats have been identified; similarly, Spp⁺ derivatives of strain CH107, which could theoretically result from uneven recombination between two sister alleles in a partially replicated chromosome, were not detected. However, recombinations among the *S. gordonii* GTF direct repeats could have significant biological consequences since the glucans produced by these different enzymes could potentially affect the ability of cells to accumulate on and/or be released from developing dental plaque surfaces (38, 39). Studies to examine the potential ecological effects of such genetic recombinations are in progress.

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