Molecular and Antigenic Characterization of a *Streptococcus oralis* Coaggregation Receptor Polysaccharide by Carbohydrate Engineering in *Streptococcus gordonii* [13]

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The coaggregation receptor polysaccharides (RPS) of *Streptococcus oralis* and related species are recognized by lectin-like adhesins on other members of the oral biofilm community and by RPS-specific antibodies. The former interactions involve β-GalNac or β-Gal containing host-like motifs in the oligosaccharide repeating units of these polysaccharides, whereas the latter involves features of these molecules that are immunogenic. In the present investigation, the molecular and corresponding structural basis for the serotype specificity of *S. oralis* ATCC 10557 RPS was determined by engineering the production of this polysaccharide in transformable *Streptococcus gordonii* 38. This involved the systematic replacement of genes in the *rps* cluster of strain 38 with different but related genes from *S. oralis* 10557 and structural characterization of the resulting polysaccharides. The results identify four unique genes in the *rps* cluster of strain 10557. These include *wef* for an α-Gal transferase, *wef* for a GalNac-1-phosphotransferase that has a unique acceptor specificity, *wefK* for an acetyl transferase that acts at two positions in the hexasaccharide repeating unit, and a novel *wzy* associated with the B1–3 linkage between these units. The serotype specificity of engineered polysaccharides correlated with the *wefI*-dependent presence of α-Gal in these molecules rather than with partial O-acetylation or with the linkage between repeating units. The findings illustrate a direct approach for defining the molecular basis of polysaccharide structure and antigenicity.

The characteristic presence of different bacteria in naturally occurring biofilm communities, such as those that form on host mucosal surfaces, raises the possibility that bacterial surface polysaccharides, in addition to their role as antigens, function as recognition molecules for biofilm development. This possibility is evident from studies of the cell wall polysaccharides on *Streptococcus oralis* and related virulids group streptococci that function as receptors for lectin-like adhesins on other members of the dental plaque biofilm community (2, 3). Structural characterization of these polysaccharides (4–9) from over 20 different streptococcal strains that coaggregate with *Actinomyces naeslundii* revealed six coaggregation receptor polysaccharides (RPS), three of which are shown in Fig. 1. The presence of a host-like motif, either GalNacβ1–3Gal (Gn) or Galβ1–3GalNacAc (G), in the oligosaccharide repeating units of these molecules accounts for recognition of RPS-bearing streptococci by GalNac- and/or Gal-reactive surface adhesins of *A. naeslundii* and various other members of the dental plaque biofilm community (10, 11). In contrast, the reactions of RPS-specific antibodies involve the common 1–3 linkage between these units. The serotype specificity of engineered polysaccharides correlated with the *wefI*-dependent presence of α-Gal in these molecules rather than with partial O-acetylation or with the linkage between repeating units. The findings illustrate a direct approach for defining the molecular basis of polysaccharide structure and antigenicity.

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2 The abbreviations used are: RPS, receptor polysaccharide; CSP, competence stimulating peptide; G motif, Galβ1–3GalNacAc; Gn motif, GalNacβ1–3Gal; HSQC, heteronuclear single quantum coherence spectroscopy; NOESY, nuclear Overhauser spectroscopy; RPS, no cell surface RPS detected by dot immunoblotting; rps, chromosomal locus for RPS biosynthesis; TOCSY, total correlation spectroscopy; GalNac, N-acetylgalactosamine; THB, Todd-Hewitt broth; ORF, open reading frame; l-Rha, l-rhamnose.

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4 M MgCl$_2$ elution from small columns of immunoadsorbent prepared by coupling partially oxidized RPS to Affi-Gel Hz (Bio-Rad) as previously described (13). RPS serotype 1-specific IgG was prepared from rabbit antiserum R26 against S. oralis 34 by elution from coupled type 1 Gn RPS (3). RPS serotype 2-specific IgG was prepared from antiserum R102 against S. gordonii 38 by elution from coupled type 2G RPS (18). RPS serotype 3-specific IgG was prepared from antiserum R98 against S. oralis 10557 (8) by elution from coupled type 3G RPS. RPS-specific IgG from antiserum R49 against S. oralis J22 (13) was also used in colony immunoblotting to detect certain RPS-producing mutants including S. gordonii GC51.

Dot immunoblotting was performed as previously described (17) to detect binding of RPS-specific antibodies to streptococci. Briefly, streptococci were harvested, washed with buffer, adjusted to uniform cell densities, and applied to nitrocellulose membranes in decreasing numbers using a Bio-Dot Microfiltration Apparatus (Bio-Rad). The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 2% skim milk and incubated with 12 ng ml$^{-1}$ RPS-specific IgG for 1 h followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h prior to development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate to detect bound antibody.

Immunodiffusion was performed as previously described with undiluted rabbit antiserum against S. oralis MC2 (18) or S. oralis 10557 (8) and 0.5 mg/ml solutions of different purified polysaccharides.

PCR Amplification and Sequencing of the Type 3G RPS Gene Cluster—The 24,688-bp DNA sequence of the S. oralis 10557 rps cluster and flanking regions (GenBank™ accession number AB289547) was assembled from the sequences of overlapping PCR products. These products were amplified from genomic DNA of strain 10557 using primers designed from sequences in rps clusters of S. gordonii 38 (13) and S. oralis J22 (17). Inverse PCR (19) was performed to extend certain sequences. The sequences were assembled and annotated using Vector NTI software (Invitrogen) and the National Center for Biotechnology Information BLAST program.

Competence-stimulating Peptides (CSP)—The gene comC and flanking regions were PCR-amplified from genomic DNA of S. oralis strains 10557 and J22 using the forward primer tArg2 (20) designed from the DNA sequence of S. pneumoniae Arg-tRNA and a reverse primer (i.e. CACCATTATTTCGAGCATAGAC) designed from a common sequence in comD of different streptococci. PCR products were purified, sequenced, and annotated as described above to identify ComC of S. oralis 10557 (i.e. MKnTEKLEQFKEVTEAEIQGDKRLPYFHFHFSLRKL) and S. oralis J22 (i.e. MKnTEKLEQFKEVTEAEIQGDKRLPYFHFHFSLRKL). The position of the putative Gly-Gly cleavage site in each sequence was used to identify the corresponding mature CSP, which is underlined. Each mature CSP was synthesized using automated 9-fluorenylmethoxy carbonyl chemistry and purified by high performance liquid chromatography (CBER Research Central, National Institutes of Health).

Construction of Streptococcal Mutant Strains—Table 1 lists the parent and mutant strains used in this study. All of the
Transformation reactions containing 50 μl of an overnight THB culture was again diluted 1/20 in fresh THB-HS and incubated at 37 °C. Following incubation for 2 h, the resulting THB containing 5% heat-inactivated horse serum (Sigma) was performed as previously described (17) with minor modifications. Briefly, PCRs contained KOD Hot Start DNA polymerase (Novagen), a template consisting of three overlapping PCR fragments (i.e. the upstream gene targeting sequence, the gene or genes of interest, and the downstream gene targeting sequence) and appropriate primers complementary to the 5’-end of the upstream gene targeting sequence and 3’-end of downstream gene targeting sequence. Individual PCR fragments were prepared by amplification of ermAM from pKSerm2 (22), spc from pDL278 (23), and various genes of interest and targeting sequences from streptococcal genomic DNA using appropriately designed primers (24). Previous results (18) have shown that insertion of the ermAM cassette, which contains its own promoter but lacks a transcriptional terminator, does not have a polar effect on the expression of downstream genes.

Transformation of S. gordonii 38 and derivatives of this strain was performed as previously described (17) with minor modifications. Briefly, an overnight THB culture was diluted 1/20 in THB containing 5% heat-inactivated horse serum (Sigma) (THB-HS). Following incubation for 2 h at 37 °C, the resulting culture was again diluted 1/20 in fresh THB-HS and incubated for 2 h at 37 °C to obtain competent cells in early log-phase. Transformation reactions containing 50 μl of competent cells, 450 μl of THB-HS, and 2 μg of transforming DNA were incubated for 2 h at 37 °C prior to plating on brain-heart infusion agar containing 5% heat-inactivated horse serum and appropriate antibiotics, which were added as needed. Transformation of S. oralis J22, S. oralis 10557, and derivatives of these strains was performed by the same procedure except that the homologous CSP was added to transformation reactions at a final concentration of 100 μg ml⁻¹. RPS-producing transformants, obtained by replacement of the ermAM or spc cassettes with genes that complemented RPS production, were identified by colony-immunoblotting with RPS-specific IgG (17). Integration of overlap extension PCR products at the expected location in the streptococcal chromosome was confirmed by PCR amplification of specific products across the upstream and downstream boundaries of the insertion using primers designed from flanking sequences that were extraneous to those used for gene targeting or by DNA sequencing of the affected region.

Purification of Polysaccharides—RPS was solubilized by mutanolysin digestion of protease-treated streptococcal cell walls and purified by gradient elution from an anion exchange column (DEAE-Sepharose Fast Flow; GE Healthcare) as previously described (8).

Structural Characterization of Polysaccharides—NMR spectra of purified polysaccharides were recorded as in previous studies (5, 17, 25) with a Bruker DRX 500 MHz spectrometer with a cryoprobe using XWINNMR as the standard acquisition software. The NMR measurements were done at 25 °C. Generally, a 10-mg sample of RPS was exchanged twice with 3 ml of 99.96% D₂O, lyophilized, and dissolved in 0.6 ml of 99.99% D₂O. The chemical shifts were recorded relative to internal acetone (IH, 2.225ppm; 13C, 31.05ppm). All of the data were processed using NMRPipe, NMRDraw, and NMRView software. Double quantum filtered homonuclear coherence spectroscopy and total correlation spectroscopy (TOCSY) were carried out to assign the scalar coupled proton of each monosaccharide residue. 13C chemical shifts were assigned by heteronuclear single quantum coherence spectroscopy (HSQC). Inter-residual linkages were determined by nuclear Overhauser spectroscopy (NOESY) with mixing times of 100 and 300 ms and in one case by long range C-H heteronuclear multiple bond coherence.

Glycosyl composition and linkage analyses were done by gas chromatography-mass spectrometry of the sugar alditol acetates and of the partially methylated alditol acetates. This work, which was performed at the Complex Carbohydrate Research Center of the University of Georgia, is described in the supplementary data.

RESULTS

Identification and Molecular Comparison of RPS Gene Clusters—The rps cluster of S. oralis 10557 (Fig. 2), which resembles those of S. oralis J22 (17) and S. oralis 34 (18), was identified downstream of dexB and two aliB-like ORFs and upstream of aliA. The 5’-end of this cluster contained the four expected regulatory genes (i.e. wzg, wzh, wzd, and wze) and the 3’-end contained the first three genes (i.e. rmlA, rmlC, and rmlB) for dTDP-α-D-Rha biosynthesis. The last gene for this pathway, rmlD, was found immediately downstream of rmlB but appeared to be transcribed in the opposite direction from a bidirectional promoter present between rmlD and aliA.

We anticipated that the unique structural features of type 3G RPS would depend on genes in the central region of the S. oralis 10557 rps cluster. To identify these genes, we sought to engineer the production of type 3G RPS in transformable S. gordonii 38. We previously (17) converted type 2G RPS of S. gordonii 38 to type 2G RPS of S. gordonii GC16 by replacing wefC and wefD in strain 38 with wefF and wefG from S. oralis J22 (Fig. 3A). In the present study, we deleted wefB from S. gordonii GC16, thereby converting type 2G RPS to type 1G RPS of S. gordonii GC27 (Fig. 3A). This deletion required two steps. The first was replacement of wefA and wefB in strain GC16 with the ermAM cassette to obtain the RPS⁻ transformant, S. gordonii GC25 (Table 1). The second was replacement of the ermAM cassette in strain GC25 with wefA from wild type S. gordonii 38 to obtain the RPS⁺ transformant, S. gordonii GC27. The 1H and 13C chemical shifts in an HSQC NMR spectrum of strain GC27 RPS (Table 2) were indistinguishable from those of previously characterized type 1G RPS of S. oralis MC2 (18); results from glycosyl composition and linkage analyses of strain GC27 RPS also support the structure shown in Fig. 2. Using the same two-step method, we then replaced selected genes in the rps cluster of strain GC27 with related genes from S. oralis 10557 (Fig. 2) and isolated the resulting polysaccharides for structural characterization.
Carbohydrate Engineering of Streptococcal RPS

The Allelic Glycosyltransferases Encoded by wefA and wefI
Differ in Donor Specificity—The presence of α-3-linked GalNAc in type 1G RPS of strain GC27 was predicted to depend on wefA (Fig. 2). Consequently, we suspected that the homologue of this gene in S. oralis 10557 accounted for the presence of α-3-linked Gal in type 3G RPS. To test this hypothesis, we constructed S. gordonii GC32 (Fig. 3A) by precise replacement of wefA in S. gordonii GC27 with the gene that is now designated wefI from S. oralis 10557, via the RPS\textsuperscript{−} transformant, S. gordonii GC30 (Table 1). Because the RPS from strain GC32 was predicted to have a novel structure, we did not expect that its NMR chemical shifts would match those of a previously described polysaccharide. Instead, tentative assignments were made by comparison of chemical shifts for domains of the structure predicted to be identical based on the genes for polysaccharide biosynthesis. Thus, chemical shifts of strain GC32 RPS (Fig. 3B and Table 2) were predicted to be identical to those of strain GC27 RPS for residues C, D, E, and F and similar for residue B with major differences only for residue A, which was predicted to be α-Gal in strain GC32 rather than α-GalNAc in strain GC27. Chemical shifts for residue A of strain GC32 RPS were predicted to be similar to those of residue A in de-O-acetylated strain 10557 RPS (S), which, as described below, is identical to strain GC35 RPS. Consideration of the data in Table 2 indicates that this scheme is reliable to approximately ± 0.02 ppm in \textsuperscript{1}H and ± 0.2 ppm in \textsuperscript{13}C. However, because chemical shift analogies do not constitute rigorous proof of a novel polysaccharide structure, the \textsuperscript{1}H chemical shift assignments for each sugar residue of strain GC32 were verified by \textsuperscript{1}H coupling coherence using coherence spectroscopy and TOCSY spectra (supplemental Fig. S1). Proton proximity and inter-residue connections were verified by NOESY spectra (supplemental Fig. S2), proving that strain GC32 RPS has a structure identical to that of type 1G RPS except for the presence of α-Gal at position A (Fig. 3C). Glycosyl composition and linkage analyses of the RPS from strain GC32 (supplemental data) are consistent with the proposed structure. The replacement of α-GalNAc with α-Gal had a dramatic effect on immunoreactivity, changing serotype 1 strain GC27 to serotype 3 strain GC32 (Fig. 3A).

In addition to distinguishing the donor specificities of WefA and WeFI, the structures of S. gordonii GC27 RPS and GC32 RPS implied that α-GalNAc and α-Gal are both potential acceptors for the subsequent Weff-dependent transfer GalNAc-1-PO\textsubscript{4}\textsuperscript{−}. In view of this, we wondered whether related Wefc, which transfers Gal-1-PO\textsubscript{4}\textsuperscript{−} to α-GalNAc in the synthesis of type 2Gn RPS (Fig. 1) could also utilize α-Gal as an acceptor. To assess this possibility, we constructed S. gordonii GC51 (Fig. 3A) by precise replacement of wefI in S. gordonii 38 with wef\textsuperscript{I} of S. oralis 10557 via the RPS\textsuperscript{−} transformant, S. gordonii GC21 (Table 1). The structure of strain GC51 RPS, which was expected to be like that of S. gordonii 38 RPS (i.e. type 2Gn) except for the presence of α-Gal at residue A, was determined using a procedure similar to that described above for strain GC32 RPS. Therefore, chemical shift assignments for S. gordonii 38 RPS were used to predict those of residues B, C, D, E, F, and G in strain GC51 RPS, whereas shifts of de-O-acetylated strain 10557 RPS, which is identical to strain GC35 RPS, were used for residue A. Examination of these data in Table 2 shows that the match of predicted and experimental chemical shifts (supplemental Fig. S3) is reasonable but not as close as that seen with strain GC32 RPS. Most notable was a 0.23 ppm discrepancy in the \textsuperscript{1}H chemical shift for the anomeric signal of residue G (α-Rha). Therefore, to verify the chemical shift and linkage assignment, the chemical shift assignments made by TOCSY (supplemental Fig. S4) and by NOESY (supplemental Fig. S5) were augmented with a long range C-H correlation by heteronuclear multiple bond coherence (supplemental Fig. S6). Glycosyl composition and linkage analyses of strain GC51 RPS (supplemental data) support the proposed structure shown in Fig. 3C. Surprisingly, strain GC51 was not stained in dot immunoblotting (Fig. 3A) following incubation with anti-2Gn/2G RPS specific IgG (i.e. R103). Thus, the reaction of this serotype-specific antibody with strain 38 must involve not only the l-Rha branch in type 2Gn RPS (9) but also adjacent α-GalNAc in this polysaccharide.

The Contribution of wzy to RPS Structure—Our model of RPS biosynthesis attributed the presence of Glcβ1-6Gal in type 1G RPS of S. gordonii GC27 to Wzy-dependent polymerization of hexasaccharide repeating units (Fig. 2). Consequently, we suspected that the presence of Glcβ1-3Gal in type 3G RPS...
Figure 3. Carbohydrate engineering of type 3G RPS in *S. gordonii*. A, the partial ORF diagram of each strain indicates the presence of genes from *S. gordonii* 38 (white arrows), *S. oralis* J22 (blue arrows), *S. oralis* 10557 (green arrows), or *ermAM* (red arrow). For dot immunoblotting, nitrocellulose membranes were spotted with decreasing numbers of each wild type or mutant streptococcal strain, incubated with RPS-specific rabbit IgG, washed, and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG and substrate. B, C-H correlation spectrum (HSQC) of RPS from strain GC32 showing the anomeric and central regions but not methyl region of the spectrum. C, novel RPS structures indicating the residue letters used for the assignment of HSQC $^1$H and $^{13}$C chemical shifts in B and Table 2.
resulted from the action of Wzy in *S. oralis* 10557. The predicted sequences of Wzy in strains 10557 and GC27 are only 19% identical (Fig. 2). To establish the role of these proteins, we constructed *S. gordonii* GC39 (Fig. 3A) by precisely replacing wzy in *S. gordonii* GC27 with wzy of *S. oralis* 10557 via the RPS transformant, *S. gordonii* GC31 (Table 1). Strain GC39 RPS was predicted to differ from strain GC27 RPS only in the linkage between β-Glc (residue C) and β-Gal (residue D) being β1–3 in the former and β1–6 in the latter structure. Accordingly, the chemical shifts of strain GC39 RPS (Table 2 and supplemental Fig. S7) matched those of strain GC27 RPS at residues A, B, E, and F and those of de-O-acetylated strain 10557 RPS (5), represented by strain GC35 in Table 2, at residues C and D. The accuracy of this assignment was verified by homonuclear coupling correlation as determined by TOCSY (supplemental Fig. S8) and proton proximity as determined by NOESY (supplemental Fig. S9), proving a structure identical to that of type 1G RPS, except for the β1–3 linkage between Glc and Gal/Glcp (Fig. 3C). Glycosyl composition and linkage analyses of strain GC39 RPS (supplemental data) confirm the proposed structure. Strains GC27 and GC39 both reacted strongly with anti-type 1Gn/1G RPS-specific IgG and weakly with anti-type 3G RPS-specific IgG in dot immunoblotting (Fig. 3A). Thus, the presence of β1–6 or β1–3 linkages between RPS repeating units did not have a noticeable effect on immunoreactivity.

**The Gene wefK Encodes an Acetyl Transferase That Acts at Two Positions in Type 3G RPS—**The novel gene wefK in the rps cluster of *S. oralis* 10557 (Fig. 2) encodes a protein with 10 transmembrane helices as defined by the SOSU1 system. The WefK sequence retrieved a group of uncharacterized proteins from the data base, including closely related WcfG, a putative acetyl transferase involved in the synthesis of several *Streptococcus pneumoniae* capsular polysaccharide serotypes (14). Like these polysaccharides, type 3G RPS of *S. oralis* 10557 is partially O-acetylated (Fig. 2). Previous estimates of this modification (i.e. 33% O-acetylation at both the 2-OH of Galf and 6-OH of GalNAc-1-P0₄⁻) were based on ¹H and ¹³C chemical shifts of acetate methyl groups and chemical shifts at both the site of O-acetylation and at adjacent sites of polysaccharides isolated from bacteria grown in complex medium supplemented with TWEEN 80 (5). Comparable estimates made in the present study for the same polysaccharide isolated from bacteria grown in THB were 10% at the 2-OH of Galf and 30% at the 6-OH of GalNAc-1-P0₄⁻. Thus, the extent of O-acetylation appeared to vary somewhat with growth conditions.

To assess the role of wefK, we inserted this gene between wxx and glf of *S. gordonii* GC27 to obtain *S. gordonii* GC38, via the RPS⁻ transformant, *S. gordonii* GC29 (Table 1).Dot immunoblotting (Fig. 3A), strain GC38 exhibited reduced anti-type 1 immuneactivity, with that of parental strain GC27. This finding, which suggested that the insertion of wefK reduced cell surface RPS production, was consistent with the relatively low yield of polysaccharide isolated from strain GC38.

The construction of *S. gordonii* GC35 (Fig. 3A) by replacement of wzy of *S. gordonii* GC32 with wzy of *S. oralis* 10557 via the RPS⁻ transformant *S. gordonii* GC34 (Table 1) provided a convenient approach for further assessing the role of wefK. The HSQC ¹H and ¹³C chemical shifts in NMR spectra of *S. gordonii* GC35 RPS, which expressed wefK and wzy from *S. oralis* 10557, were indistinguishable from those of de-O-acetylated type 3G RPS prepared by chemical de-O-acetylation (5). Glycosyl composition and linkage analyses of strain GC35 RPS (supplemental data) also agree with the expected structure. We then constructed *S. gordonii* GC37 (Fig. 3A) by inserting wefK between wxx and glf of *S. gordonii* GC35, via the RPS⁻ transformant, *S. gordonii* GC36 (Table 1). NMR spectra recorded for strain...
### TABLE 2

Residue by residue comparison of HSQC $^1$H and $^{13}$C chemical shifts for the RPS of *S. gordonii* 3B and mutant strains

| Strain   | Residue | H-1, C-1 | H-2, C-2 | H-3, C-3 | H-4, C-4 | H-5, C-5 | H-6, H-6', C-6 | NAc, CH$_3$ |
|----------|---------|----------|----------|----------|----------|----------|----------------|-------------|
| Sg 38    | GalNAc (A) | 5.180    | 4.233    | 3.973    | 4.078    | 4.330    | 4.01, 4.07   | 2.087, 23.12 |
| GC27     | GalNAc (A) | 5.071    | 4.222    | 4.012    | 4.063    | 4.215    | 4.315, 4.398 | 5.047, 4.039 |
| GC32     | Gal (A)    | 9.15     | 5.127    | 3.851    | 3.963    | 4.051    | 4.344, 3.980 | 4.024, 65.92 |
| GC51     | Gal (A)    | 9.67     | 69.13    | 70.11    | 69.72    | 70.50    | 65.42         |
| GC39     | GalNAc (A) | 5.148    | 9.718    | 68.77    | 69.89    | 69.52    | 70.37, 64.78 |
| GC53     | Gal (A)    | 5.127    | 9.67     | 69.13    | 70.11    | 69.72    | 70.50         |
| Sg 38    | Rha (B)    | 5.070    | 4.225    | 4.015    | 4.066    | 4.354    | 4.396, 4.042 | 2.057, 23.04 |
| GC27     | Rha (B)    | 9.50     | 5.058    | 68.35    | 68.94    | 70.70    | 65.42         |
| GC32     | Rha (B)    | 9.13     | 100.51   | 73.05    | 79.63    | 71.75    | 73.42         |
| GC51     | Rha (B)    | 9.76     | 101.36   | 73.46    | 79.11    | 71.67    | 73.43         |
| GC39     | Rha (B)    | 9.38     | 101.55   | 68.35    | 78.70    | 71.28    | 73.04         |
| GC35     | Rha (B)    | 9.38     | 101.55   | 68.35    | 78.70    | 71.28    | 73.04         |
| Sg 38    | Glc (C)    | 9.489    | 103.53   | 74.02    | 76.83    | 78.07    | 75.60         |
| GC27     | Glc (C)    | 9.412    | 103.38   | 73.79    | 76.04    | 77.16    | 75.35         |
| GC32     | Glc (C)    | 9.410    | 103.70   | 74.21    | 76.36    | 77.53    | 75.58         |
| GC51     | Glc (C)    | 9.497    | 103.74   | 74.04    | 76.90    | 78.15    | 75.67         |
| GC39     | Glc (C)    | 9.613    | 102.92   | 73.82    | 76.17    | 77.53    | 75.58         |
| GC35     | Glc (C)    | 9.615    | 102.92   | 73.82    | 76.17    | 77.53    | 75.58         |
| Sg 38    | Galβ (D)   | 9.06     | 108.66   | 81.78    | 77.53    | 83.93    | 70.51         |
| GC27     | Galβ (D)   | 9.061    | 108.36   | 81.51    | 77.41    | 83.61    | 70.23         |
| GC32     | Galβ (D)   | 9.059    | 108.78   | 81.83    | 77.73    | 83.98    | 70.50         |
| GC51     | Galβ (D)   | 9.067    | 108.57   | 81.87    | 77.55    | 84.01    | 70.84         |
| GC39     | Galβ (D)   | 9.089    | 108.58   | 80.46    | 85.34    | 83.00    | 71.28         |
| GC35     | Galβ (D)   | 9.088    | 108.58   | 80.46    | 85.34    | 83.00    | 71.28         |
| Sg 38    | GalNAc (E) | 9.657    | 104.01   | 53.37    | 71.63    | 68.78    | 74.58         |
| GC27     | Galβ (E)   | 9.527    | 105.09   | 71.16    | 73.06    | 69.35    | 74.28         |
| GC32     | Galβ (E)   | 9.527    | 105.09   | 71.16    | 73.06    | 69.35    | 74.28         |
| GC51     | GalNAc (E) | 9.645    | 105.09   | 71.16    | 73.06    | 69.35    | 74.28         |
| GC39     | Galβ (E)   | 9.533    | 105.46   | 71.48    | 73.43    | 69.72    | 74.60         |
| GC35     | Galβ (E)   | 9.529    | 105.46   | 71.48    | 73.43    | 69.72    | 74.60         |
| Sg 38    | Gal (F)    | 9.495    | 96.58    | 68.02    | 79.68    | 70.05    | 72.38         |
| GC27     | GalNAc (F) | 9.486    | 94.93    | 49.23    | 77.26    | 69.15    | 72.43         |
| GC32     | GalNAc (F) | 9.489    | 95.30    | 49.60    | 77.73    | 69.52    | 72.65         |
| GC51     | Gal (F)    | 9.46     | 96.72    | 68.98    | 79.68    | 70.12    | 72.40         |
| GC39     | GalNAc (F) | 9.487    | 95.30    | 49.41    | 77.92    | 69.33    | 72.65         |
| GC35     | GalNAc (F) | 9.486    | 95.30    | 49.41    | 77.92    | 69.33    | 72.65         |
| Sg 38    | Rha (G)    | 5.09     | 100.92   | 71.29    | 71.10    | 72.75    | 69.47         |
| GC51     | Rha (G)    | 5.274    | 101.58   | 71.06    | 71.00    | 72.88    | 69.46         |

* Sg 38 denotes type 2Gn RPS from *S. gordonii* 38 (9).
Antiserum against type 1G RPS (Fig. 4, left panel), serotype-specific immunoprecipitation was noted with RPS from wefI-containing S. gordonii GC32 (well 4) and with type 3G RPS from S. gordonii GC37 (well 5) or S. oralis 10557 (well 6). The weak reactions seen with other polysaccharides (i.e. those in wells 1, 2, and 3) were not serotype-specific. Thus, RPS serotype 3 immunoreactivity reflected the wefI-dependent presence of α-Gal in different polysaccharides rather than partial O-acetylation or the β1–3 linkage between RPS repeating units.

The Allelic Glycosyl-1-Phosphotransferases Encoded by wefF and wefG Differ in Acceptor Specificity—In view of the subtle difference in the acceptor specificities of WefC and WefH (Fig. 1) for linear versus branched structures, we wondered whether a similar difference might exist between Weff of S. oralis J22 and the homologue of this transferase in S. oralis 10557 (i.e. Weff in Fig. 2). To examine this possibility, we tested weff for its ability to complement type 2G RPS production in S. oralis J22 (Fig. 5). Initially, we replaced weff of strain J22 with the spc cassette to obtain the RPS⁻ transformant, S. oralis MC10. We then replaced the spc cassette in strain MC10 with weff genetically linked to the ermA cassette. The presence of ermA did not have a polar effect on the expression of downstream genes. The resulting RPS⁻ transformant, S. oralis MC11 reacted weakly with anti-type1Gn/1G RPS-specific IgG (Fig. 5). Consistent with this reaction, the HSQC spectra of S. oralis MC11 RPS was identical to that of type 1G RPS (18), with no evidence for the presence of i-Rha branches. This finding suggested that the action of Weff was limited to the relatively small amount of linear acceptor that remained available in the presence of WeffB. Finally, we constructed S. oralis MC12 (Fig. 5) by inserting weff (linked to the nonpolar ermA cassette) between weff and wefg of parental S. oralis J22. Strong immunostaining of strain MC12 was noted with either type 1Gn/1G RPS or 2Gn/2G RPS-specific IgG, thereby suggesting the production of a hybrid polysaccharide from the action of both Weff and Wefg in this strain. Thus, the acceptor specificities of these GalNac-1-phosphotransferases are distinct.

The Predicted Properties of Truncated wzy—The 116-amino acid coding sequence of the small ORF located between wefg and wzy of S. oralis 10557 (Fig. 2) is ~53% identical to the N-terminal regions of Wzy from S. oralis 34, S. gordonii 38, or S. oralis J22. To determine whether the truncated ORF in strain 10557 represents non essential DNA, we precisely deleted this region to obtain strain TC3, via the RPS⁻ transformant, strain TC2 (Table 1). As anticipated, strain TC3 and wild-type S. oralis 10557 were indistinguishable in dot immunoblotting performed with anti-type 3G RPS-specific IgG (results not shown).

We then wondered whether truncated wzy-like ORFs similar to the one in S. oralis 10557 occur in other type 3G RPS-producing isolates. To address this question we PCR-amplified and sequenced the wefg-wzy region in each of four seemingly unrelated type 3G RPS-producing isolates (Table 1), S. oralis SK23, S. gordonii SK120, S. oralis 4477, and S. oralis H127. Interestingly, these strains all contained truncated wzy-like coding sequences, (GenBank™ accession number AB301711, AB301712, AB301713, and AB301714, respectively). The 2922-bp sequence of the wefg-wzy region in strain 10557, including the 780-bp sequence between these genes, was iden-
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**S. oralis J22** (2G)  

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\[ \text{wchF} \rightarrow \text{wefA} \rightarrow \text{wefB} \rightarrow \text{wefC} \rightarrow \text{wefD} \]
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**S. oralis MC10**  

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\[ \text{wchF} \rightarrow \text{wefA} \rightarrow \text{wefB} \rightarrow \text{spc} \rightarrow \text{wefC} \rightarrow \text{wefD} \]
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**S. oralis MC11** (1G)  

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\[ \text{wchF} \rightarrow \text{wefA} \rightarrow \text{wefB} \rightarrow \text{ermAM} \rightarrow \text{wefC} \rightarrow \text{wefD} \]
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**S. oralis MC12**  

```
\[ \text{wchF} \rightarrow \text{wefA} \rightarrow \text{wefB} \rightarrow \text{ermAM} \rightarrow \text{wefC} \rightarrow \text{wefD} \]
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**FIGURE 5.** Dot immunoblots of wild type and mutant streptococci with anti-type 1Gn/1G and anti-type 2Gn/2G RPS-specific antibodies showing a difference in the acceptor specificities of the GalNAc-1-phosphotransferases encoded by *wefF* of *S. oralis* 10557 and allelic *wefF* of *S. oralis* 10557. ORF diagrams of different strains identify genes from *S. oralis* J22 (blue arrows), *S. oralis* 10557 (green arrows), spc (orange arrow), or ermAM (red arrow). Strain MC11, obtained by replacing *wefF* in *S. oralis* J22 with *wefF* (linked to *ermAM*), produced a relatively small amount of type 1G RPS, which was identified by HSQC spectra of the isolated polysaccharide.

**DISCUSSION**

The results of the present study associate the distinct structural and biological properties of *S. oralis* 10557 type 3G RPS and types 1Gn, 2Gn, and 2G RPS of other strains with a relatively small number of genes for different glycosyl or glycosyl-1-phosphotransferases (Fig. 6). Three of these (i.e. *wchA*, *wchF*, and *wefF*) account for the common presence of Rhaβ1-4Glc at one end and Galβ at the other end of each RPS repeating unit (13), whereas nine others determine the variable features of these polysaccharides. Four distinct RPS serotypes are associated with the presence of allelic *wefI* or *wefA* and the presence or absence of downstream *wefB* in different strains. In the absence of *wefB*, the *wefA*- or *wefI*-dependent transfer of α-GalNAc or α-Gal to Rhaβ1-4Glc leads to the formation of linear serotype 1 or 3 polysaccharides, respectively. In contrast, the apparent action of *WefA* or *WefI* followed by that of *WefB* (i.e. synthesis of the L-Rha branch) yields branched polysaccharides that react as different serotypes. Synthesis of the recognition motifs in these polysaccharides depends on genes from two allelic groups. One group encodes the glycosyl-1-phosphotransferases (i.e. *WefF*, *WefE*, *WefC*, and *WefH*) that add Gal-1-PO₄⁻ or GalNAc-1-PO₄⁻ to the branched or linear acceptors associated with different serotypes. The other group encodes *WegF* and *WefD*, which catalyze the subsequent β-3 transfer of Gal or GalNAc, respectively, completing the recognition motifs in these polysaccharides. The *WefE*-dependent transfer of Galβ to terminal β-Gal or β-GalNAc sets the stage for Wzx-dependent transport and Wzy-dependent polymerization by allelic polymerases that join the conserved Glc and Galβ ends of adjacent repeats through β1-6 or β1-3 linkages. Thus, the distinct structural and biological properties of these polysaccharides depend to a considerable extent on the complementary activities of different allelic transferases and polymerases.

We previously identified several transferases involved in RPS biosynthesis (Fig. 1) by the effects of the corresponding genes on the structures of polysaccharides produced by transformable *S. gordonii* 38 (17, 18). We have now extended this approach to type 3G RPS of *S. oralis* 10557. Initially, we converted the type 2Gn rps cluster of *S. gordonii* 38 to a type 1G rps cluster in *S. gordonii* GC27 and then transformed this strain and various intermediates with genes from *S. oralis* 10557 to engineer the production of type 3G RPS. The results obtained firmly establish the donor specificities of allelic *WefA* and *WefI*, the different linkage specificities of Wzy in strain 38 and strain 10557 and identify *WefK* as an acetyl transferase that acts at

**RPS specific IgG**

![Diagram of different strains identifying genes from S. oralis J22 (blue arrows), S. oralis 10557 (green arrows), spc (orange arrow), or ermAM (red arrow). Strain MC11, obtained by replacing wefF in S. oralis J22 with wefF (linked to ermAM), produced a relatively small amount of type 1G RPS, which was identified by HSQC spectra of the isolated polysaccharide.**

![Image of Dot immunoblots with anti-type 1Gn/1G and anti-type 2Gn/2G RPS-specific antibodies showing a difference in the acceptor specificities of the GalNAc-1-phosphotransferases encoded by wefF of S. oralis 10557 and allelic wefF of S. oralis 10557.](image-url)
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**FIGURE 6. Proposed molecular basis of RPS structure and function based on the structures of polysaccharides synthesized from RPS gene clusters of wild type or genetically engineered strains of streptococci.** The red arrows indicate the sites for linkage of donor to acceptor.

| Protein | Donor | Linkage | Acceptor | Strain          |
|---------|-------|---------|----------|-----------------|
| WefG    | Gal   | α1-3    | GalNAc   | 10557, GC32     |
| WefI    | GalNAc| α1-3    | GalNAc   | 38              |
| WefB    | Rhα   | α1-2    | GalNAc   | GC51            |
| WefC    | GalNAc1-PO₄⁺ | α1-6 | GalNAc1-3Rhα1-4Glc1 | J22, MC12, GC16 |
| WefI    | GalNAc1-PO₄⁺ | α1-6 | GalNAc1-3Rhα1-4Glc1 | MC2, GC27, GC32 |
| WefJ    | GalNAc1-PO₄⁺ | α1-6 | GalNAc1-3Rhα1-4Glc1 | 10557 |
| WefC    | GalNAc1-PO₄⁺ | α1-6 | GalNAc1-3Rhα1-4Glc1 | MC11, MC12     |
| WefI    | GalNAc1-PO₄⁺ | α1-6 | GalNAc1-3Rhα1-4Glc1 | 34, GC48        |
| WefD    | Gal    | β1-3    | GalNAc   | J22             |
| WefI    | GalNAc | β1-3    | GalNAc   | GC19            |

The ability to alter streptococcal polysaccharide production by genetic transformation, which dates back to the discovery of the biological role of DNA (28), was utilized in previous studies of *S. pneumoniae* capsular polysaccharide serotype 19 biosynthesis (29). Refinements of this approach in our studies of RPS biosynthesis include the precise replacement of individual genes in the *rps* cluster of one strain with complementary genes from other strains and structural characterization of the resulting polysaccharides. The NMR spectra of such polysaccharides isolated from mutanolysin cell wall digest by anion exchange column chromatography indicate that a homogeneous oligosaccharide repeating subunit comprises at least 80% of each RPS sample. However, unassigned resonances with variable intensities in the 5–20% range are also typically present in HSQC spectra, such as those of strain GC32 RPS (Fig. 3b) and strain GC51 RPS (supplemental Fig. S3). Glycosyl composition and linkage analyses of these and other RPS samples in the present study (supplemental data) revealed the expected sugars as well as small amounts of GlcNAc, terminal Glc, and Rha in linkages that are not expected from the RPS structure determined by NMR. Thus, the unassigned resonances noted in NMR spectra of RPS preparations most likely reflect the presence of extraneous cell wall polysaccharides, such as the putative rhamnose-glucose polysaccharide of *S. gordonii* (13). Although it remains to be determined whether such polysaccharides are linked to RPS through small fragments of peptidoglycan, a promising approach for their elimination involves deletion of the corresponding genes, thereby creating strains that only produce RPS. Studies to examine this possibility are underway.

In previous studies, we showed that α-GalNAc is the immunodominant sugar of RPS serotype 1 polysaccharides (12) and also that the antigenic difference between serotype 1 and serotype 2 polysaccharides depends on the presence or absence of L-Rha branches (9). We have now established the molecular and corresponding structural basis for the distinct serotype specificity of type 3G RPS by engineering the production of this polysaccharide from type 1G RPS of *S. gordonii* GC27 (Figs. 2 and 3). Surprisingly, we converted type 1G RPS to a polysaccharide that was antigenically identical to type 3G RPS of *S. oralis* 10557 simply by swapping *wefA* and *wefI*, thereby changing α-GalNAc in type 1G RPS to α-Gal in the RPS of strain GC32. In contrast, polysaccharides obtained by changing the WefK-dependent linkage from β1–6 to β1–3 or by the WefK-dependent introduction of O-acetyl groups at two positions reacted like type 1G RPS. In related experiments, swapping *wefA* for *wefI* changed α-GalNAc in type 2Gn RPS of *S. gordonii* 38 to α-Gal in the branched RPS of *S. gordonii* GC51. We expected that this structural change would not have a dramatic effect on antigenicity because of the common presence of immunodominant L-Rha branches (9) in the polysaccharides of these strains. Instead, RPS serotype 2-specific IgG, which reacted with strain 38 as expected, failed to react with strain GC51. Thus, the WefA-dependent presence of α-GalNAc and WefI-dependent presence of α-Gal appear to be critical for the synthesis of different linear as well as branched RPS serotypes (*i.e.* linear serotypes 1 and 3 and branched serotypes 2 and GC51), each of which can occur in association with either of two receptor types (Fig. 6).

RPS-bearing streptococci, which include strains of *S. sanguinis*, *S. gordonii*, and *S. oralis*, are thought to fill specific niches within the host oral environment. Importantly, this environment includes the characteristic biofilm communities that are passed from one generation to the next in each host species. Within this context, the structural diversity seen in different types of RPS, like that seen in cell surface glycans of higher multicellular organisms (30, 31), can be attributed to a wide range of selection pressures associated with both the negative and positive consequences of specific recognition events. Thus, in theory, the present day repertoire of RPS serotypes can be traced back to the survivors of past lethal encounters between...
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bacteria and the host immune system or perhaps bacteriophage. In contrast, the presence of host-like features in these polysaccharides may depend at least in part on the selective advantage gained from adhesin-mediated recognition of RPS-bearing streptococci by other commensal species. Such interactions, which result in intimate associations between different bacteria, may be a prerequisite for the establishment of mutualism in biofilm communities (3, 33). If so, the evolution of these communities may be associated with the evolution of different RPS types. In addition to the presently considered types, such polysaccharides include type 4Gn RPS, which contains ribitol-5-phosphate but not Glc or L-Rha (6, 8) and the RPS of S. oralis ATCC 55229 (34), which has putative Rha α1–2Rha recognition motifs (35). The evolutionary history of these polysaccharides, although not defined, may parallel that of the human oral environment. In this regard, the identification of what appears to be a remnant of the same ancestral wzy in the rps clusters of five independent type 3G RPS-producing strains (Fig. 6) suggests that the replacement of this gene with present day wzy is not a recent event. The association of other specific genes with the unique features of type 3G RPS and related structural types provides additional molecular markers for tracing the evolution of these polysaccharides in the biofilm communities of man and related species. The information gained may well provide insight into the role these polysaccharides play in biofilm development and their coevolution with the host oral environment.

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