THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 18, pp. 12654–12664, May 2, 2008
Printed in the U.S.A.

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

Received for publication, February 21, 2008 Published, JBC Papers in Press, February 25, 2008, DOI 10.1074/jbc.M801412200

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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 *wefI* for an α-Gal transferase, *wefJ* 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 β1–3 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 (1), 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 viridans 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),[2] three of which are shown in Fig. 1. The presence of a host-like motif, either GalNAcβ1–3Gal (Gn) or Galβ1–3GalNAc (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 L-rhamnose (L-Rha)-branched region in serotype 2 polysaccharides (9) or α-GalNAc in serotype 1 polysaccharides (12). Thus, the determinants of RPS serotype (*i.e.* 1, 2, 3, etc.) and receptor type (*i.e.* Gn or G) appear to be distinct.

Studies of RPS structure and function were extended to the molecular level by identification of the chromosomal locus (*rps*) of *Streptococcus gordonii* 38 for type 2Gn RPS biosynthesis (13). The *rps* cluster of this strain was found to contain 14 genes, including seven for putative glycosyltransferases. The first two, *wchA* and *wchF*, were associated with the presence Rhaβ1–4Glc in these polysaccharides (Fig. 1) based on studies (14–16) of similar genes for capsular polysaccharide biosynthesis in closely related *Streptococcus pneumoniae*. Genes for other glycosyl and glycosyl-1-phosphotransferases were identified by their effects on RPS structure in gene replacement experiments. Thus, replacement of the genes designated *wefC* and *wefD* in strain 38 with those designated *wefF* and *wefG* in *S. oralis* J22 switched RPS production from type 2Gn to 2G (17). Moreover, the replacement of *wefC* with *wefF* or *wefD* with *wefG* resulted in the synthesis of modified recognition motifs, either GalNAcβ1–3GalNAc or Galβ1–3Gal, respectively, thereby

* This work was supported in part by the Intramural Research Program of the NIDCR, National Institutes of Health, by Grant 96 from the Keiryokai Research Foundation, by Grants-in-Aid for Promoting Technological Seeds and a High-Tech Research Project (2005–2009) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. Y.), and by Grant 02-12702 from the National Science Foundation (to C. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data and supplemental Figs. S1–S9.

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[2] The abbreviations used are: RPS, receptor polysaccharide; CSP, competence stimulating peptide; G motif, Galβ1–3GalNAc; 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.

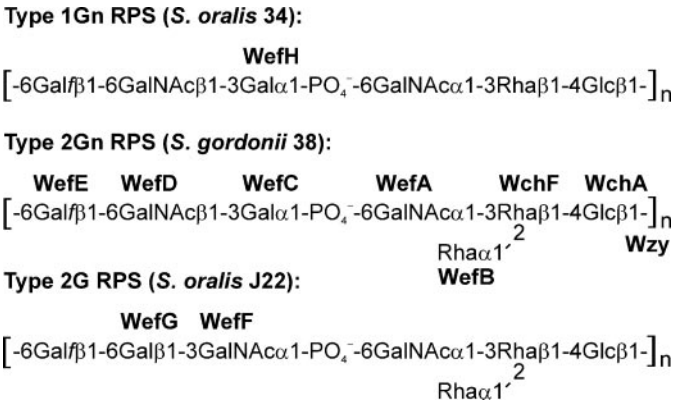


FIGURE 1. **Types of RPS produced by *S. oralis* 34, *S. gordonii* 38, and *S. oralis* J22 indicating the molecular basis of RPS structure.** Synthesis of types 1Gn and 2G RPS depends on genes that are complementary to those in strain 38 except as indicated for the transferases associated with the unique structural features of these polysaccharides.

firmly establishing the donor specificity of each encoded transferase (Fig. 1). In other studies, deletion of *wefB* eliminated L-Rha branches, converting types 2Gn and 2G RPS to linear types 1Gn and 1G, respectively (18). Further results gained from studies of genetic complementation distinguished the GalNAc-1-phosphotransferases encoded by downstream *wefC* of *S. gordonii* 38 and *wefH* of *S. oralis* 34 by a subtle difference in acceptor specificity. Both transferases acted on the linear acceptor formed in the absence of *wefB*, but only WefC acted on the branched acceptor formed in the presence of *wefB*. Thus, genetic engineering of RPS gene clusters and structural characterization of the resulting polysaccharides provides an approach for distinguishing closely related genes such as *wefH*, *wefC*, and *wefF* by differences in either the donor or the acceptor specificities of their encoded transferases.

Molecular studies of RPS structure and function have now been extended to type 3G RPS of *S. oralis* ATCC 10557 (5), a polysaccharide that is structurally related but antigenically distinct from those described above. In the present communication, we describe the *rps* cluster of *S. oralis* 10557 and the utilization of selected genes from this strain to engineer the production of type 3G RPS in transformable *S. gordonii* 38. The results associate the unique structural features of this polysaccharide with four presently described genes and identify one of these as a principal determinant of RPS serotype specificity. These findings, which complete the comparative molecular characterization of a major RPS group (8), provide the necessary basis for tracing the evolution of these polysaccharides as recognition molecules for biofilm formation in the host oral environment.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The wild type and mutant streptococci used in this study (see Table 1) were grown at 37 °C in Todd-Hewitt broth (THB) or brain-heart infusion (Difco Laboratories). These media were supplemented with erythromycin at 10 μg ml^{-1} or spectinomycin at 250 μg ml^{-1} as needed for the maintenance of antibiotic-resistant streptococcal strains.

Antibodies and Immunochemical Methods—RPS serotype-specific antibodies were affinity-purified from selected sera by 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-specifc IgG was prepared from rabbit antiserum R26 against *S. oralis* 34 by elution from coupled type 1Gn 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^{TM} 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. pneumonia* 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.* MKNTEKLEQFKEVTEAELQEIRGGDKRLPYFFKHLFSNRTK) and *S. oralis* J22 (*i.e.* MKNTEKLEQFKKVTEAELQEIRGGEIRKENNFLFYFFKRK). 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

mutant strains were prepared following previously described molecular methods (17). This involved the transformation of *S. oralis* or *S. gordonii* parental strains with PCR products that contained the *ermAM* or *spc* cassette or various intact genes from *S. gordonii* 38 or *S. oralis* 10557 flanked by 0.5–1-kb gene targeting sequences for homologous recombination. Transforming DNA was prepared by overlap extension PCR (21) performed as previously described (17). 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% D_2O, lyophilized, and dissolved in 0.6 ml of 99.99% D_2O. The chemical shifts were recorded relative to internal acetone (^{1}H, 2.225ppm; ^{13}C, 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. ^{13}C 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 supplemental 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-L-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 2Gn 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. 3*A*). 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. 3*A*). 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 ^{1}H and ^{13}C 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.

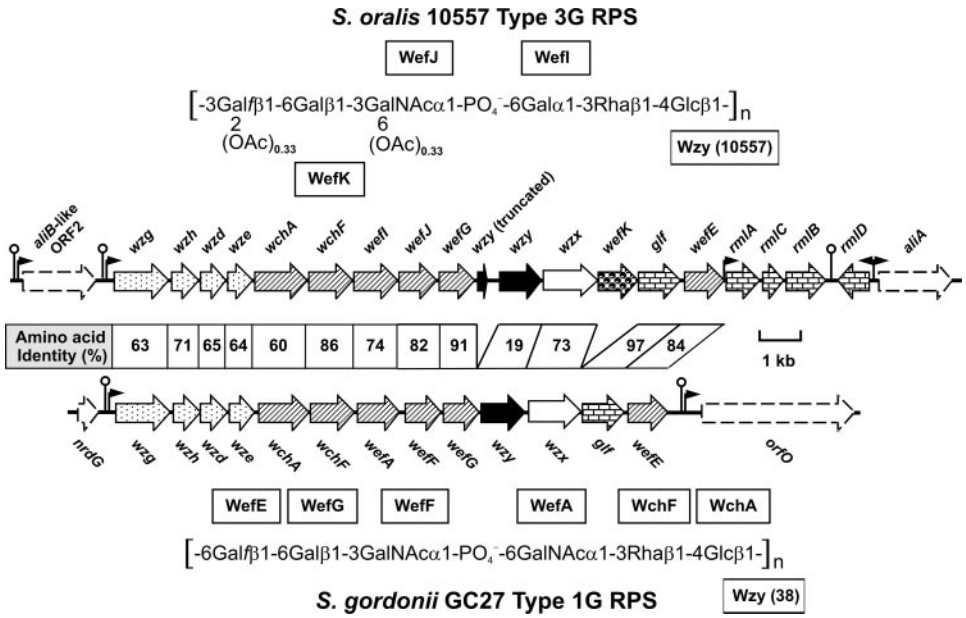
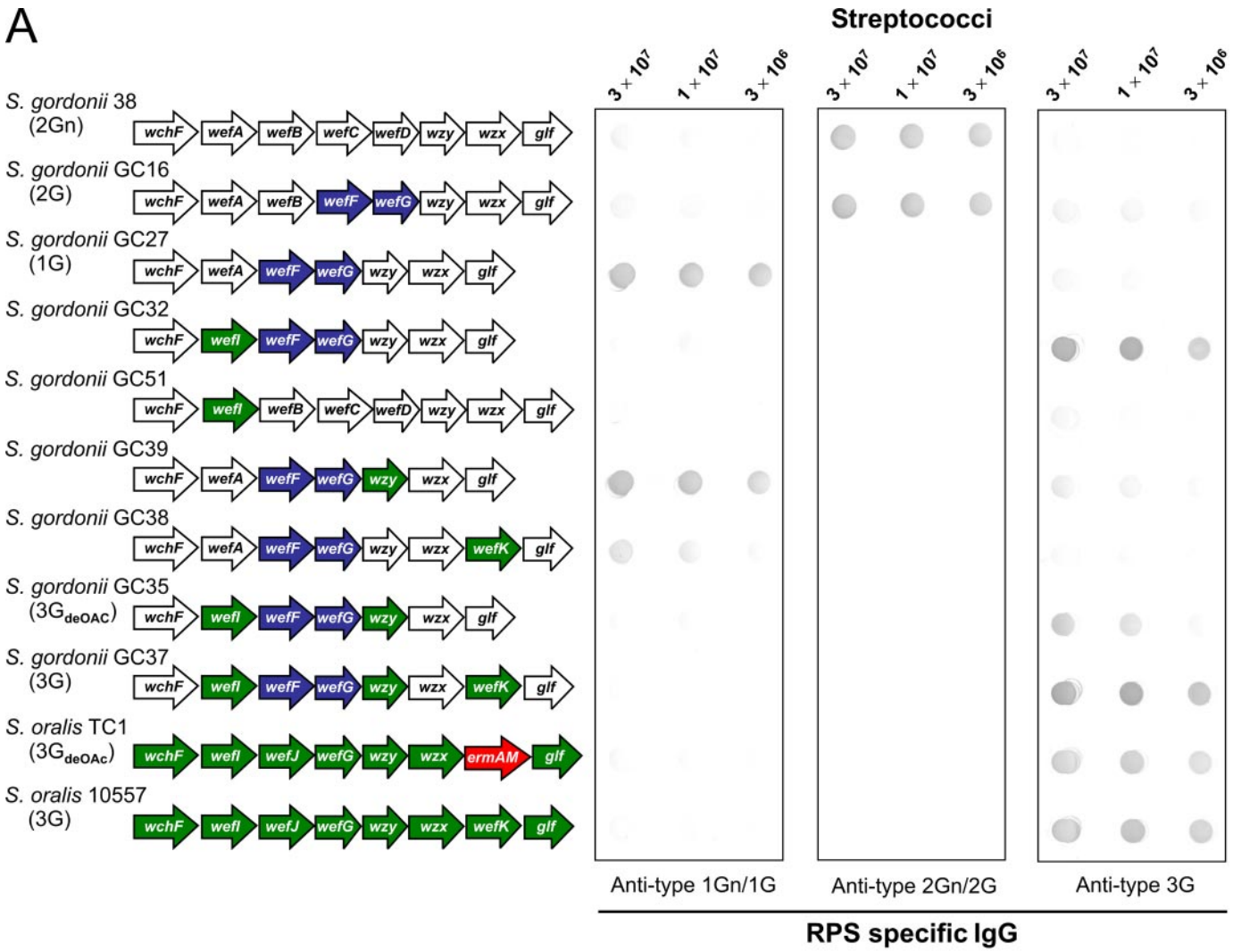


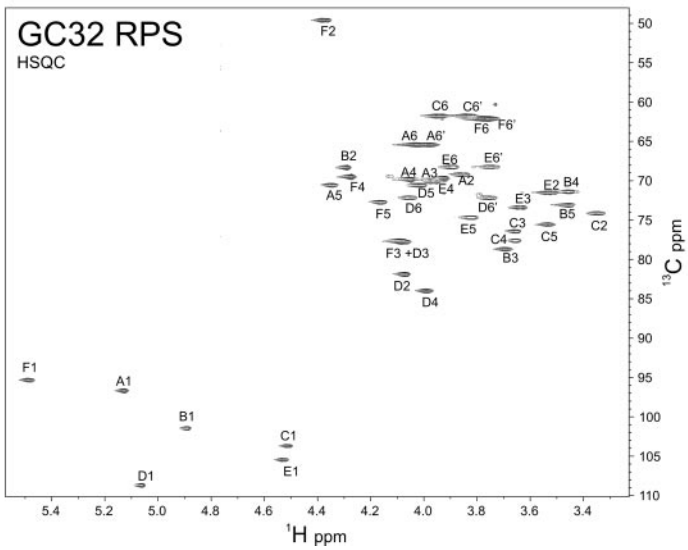
FIGURE 2. **Molecular comparison of *S. oralis* 10557 type 3G RPS with *S. gordonii* GC27 type 1G RPS showing genes for common regulatory proteins (*arrow* with *dots*), glycosyl or glycosyl-1-phosphotransferases (*hatched arrow*), polymerases (*black arrow*), flippases (*white arrow*), enzymes for nucleotide sugar biosynthesis (*arrow* with *bricks*), and in the case of strain 10557, an acetyl transferase (*arrow* with *circles*).** Synthesis of type 3G RPS depends on genes that are complementary to those in strain GC27 except as indicated for the transferases and polymerase associated with the unique structural features of type 3G RPS.

The Allelic Glycosyltransferases Encoded by wefA and wefI Differ in Donor Specificity—The presence of α1–3-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 α1–3-linked Gal in type 3G RPS. To test this hypothesis, we constructed *S. gordonii* GC32 (Fig. 3*A*) by precise replacement of *wefA* in *S. gordonii* GC27 with the gene that is now designated *wefI* from *S. oralis* 10557, via the RPS$^-$ 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. 3*B* 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 (5), 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 ^{1}H and ± 0.2 ppm in ^{13}C. However, because chemical shift analogies do not constitute rigorous proof of a novel polysaccharide structure, the ^{1}H chemical shift assignments for each sugar residue of strain GC32 were verified by ^{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. 3*C*). 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. 3*A*).

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 GalNAcα-1-PO$_4^-$. In view of this, we wondered whether related WefC, which transfers Galα-1-PO$_4^-$ 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. 3*A*) by precise replacement of *wefA* in *S. gordonii* 38 with *wefI* of *S. oralis* 10557 via the RPS$^-$ 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 ^{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. 3*C*. Surprisingly, strain GC51 was not stained in dot immunoblotting (Fig. 3*A*) 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*f* 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*f* in type 3G RPS





C

GC32 RPS

(D) (E) (F) (A) (B) (C)

$[\text{-6Gal}f\beta\text{1-6Gal}\beta\text{1-3GalNAc}\alpha\text{1-PO}_4^-\text{-6Gal}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$

GC51 RPS

$[\text{-6Gal}f\beta\text{1-6GalNAc}\beta\text{1-3Gal}\alpha\text{1-PO}_4^-\text{-6Gal}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$

(G) Rhaα1´²

GC39 RPS

$[\text{-3Gal}f\beta\text{1-6Gal}\beta\text{1-3GalNAc}\alpha\text{1-PO}_4^-\text{-6GalNAc}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$

GC35 RPS

$[\text{-3Gal}f\beta\text{1-6Gal}\beta\text{1-3GalNAc}\alpha\text{1-PO}_4^-\text{-6Gal}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$

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.

TABLE 1
Streptococcal strains used in this study

Strain	Description	Source
S. oralis 10557	ATCC 10557, human subacute bacterial endocarditis isolate (36), type 3G RPS (5)	Ref. 37
S. oralis TC1	*S. oralis* 10557 containing *ermAM* in place of *wefK*, RPS^{+b}	This study
S. oralis TC2	*S. oralis* 10557 containing *ermAM* in place of the truncated *wzy* and *wzy*, RPS^{-b}	This study
S. oralis TC3	*S. oralis* TC2 containing *wzy* of *S. oralis* 10557 in place of *ermAM*, RPS^{+}	This study
S. gordonii 38	type 2Gn RPS (9,13)	Ref. 37
S. gordonii GC16	*S. gordonii* 38 containing *wefF* and *wefG* of *S. oralis* J22 in place of *wefC* and *wefD*, type 2G RPS	Ref. 17
S. gordonii GC25	*S. gordonii* GC16 containing *ermAM* in place of *wefA* and *wefB*, RPS^{-}	This study
S. gordonii GC27	*S. gordonii* GC25 containing *wefA* of *S. gordonii* 38 in place of *ermAM*, type 1G RPS	This study
S. gordonii GC30	*S. gordonii* GC27 containing *ermAM* in place of *wefA*, RPS^{-}	This study
S. gordonii GC32	*S. gordonii* GC30 containing *wefI* of *S. oralis* 10557 in place of *ermAM*, RPS^{-}	This study
S. gordonii GC21	*S. gordonii* 38 containing *ermAM* in place of *wefA*, RPS^{-}	Ref. 18
S. gordonii GC51	*S. gordonii* GC21 containing *wefI* of *S. oralis* 10557 in place of *ermAM*, RPS^{+}	This study
S. gordonii GC31	*S. gordonii* GC27 containing *ermAM* in place of *wzy*, RPS^{-}	This study
S. gordonii GC39	*S. gordonii* GC31 containing *wzy* of *S. oralis* 10557 in place of *ermAM*, RPS^{+}	This study
S. gordonii GC29	*S. gordonii* GC27 containing *ermAM* in place of *wzx*, RPS^{-}	This study
S. gordonii GC38	*S. gordonii* GC29 containing *wzx* of *S. gordonii* 38 and *wefK* of *S. oralis* 10557 in place of *ermAM*, RPS^{+}	This study
S. gordonii GC34	*S. gordonii* GC32 containing *ermAM* in place of *wzy*, RPS^{-}	This study
S. gordonii GC35	*S. gordonii* GC34 containing *wzy* of *S. oralis* 10557 in place of *ermAM*, RPS^{+}	This study
S. gordonii GC36	*S. gordonii* GC35 containing *ermAM* in place of *wzx*, RPS^{-}	This study
S. gordonii GC37	*S. gordonii* GC36 containing *wzx* of *S. gordonii* 38 and *wefK* of *S. oralis* 10557 in place of *ermAM*, type 3G RPS	This study
S. oralis J22	Wild type strain, type 2G RPS (4,17)	Ref. 37
S. oralis MC10	*S. oralis* J22 containing *spc* in place of *wefF*, RPS^{-}	This study
S. oralis MC11	*S. oralis* MC10 containing *ermAM* linked to *wefJ* of *S. oralis* 10557 in place of *spc*, type 1G RPS	This study
S. oralis MC12	*S. oralis* J22 containing *ermAM* linked to *wefJ* of *S. oralis* 10557 between *wefF* and *wefG*, RPS^{+}	This study
S. gordonii SK120	PB179, human oral isolate (38), type 3G RPS (8)	Ref. 39
S. oralis SK23	PB182, human oral isolate (38), type 3G RPS (8)	Ref. 39
S. oralis H127[a]	Human periodontal pocket (32), type 3G RPS (8)	Ref. 37
S. oralis 4477	Human (infant) oral isolate, type 3G RPS (C. A. Bush, unpublished)	M. Cole

[a] *S. mitis* H127 (8) has been reclassified as *S. oralis* (M. Kilian, personal communication).
[b] RPS^{+} or RPS^{-} indicates that cell surface RPS was either detectable or not detectable, respectively, by dot immunoblotting with RPS-specific IgG.

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. 3*A*) 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*f* (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*f* (Fig. 3*C*). 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. 3*A*). 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 SOSUI system. The WefK sequence retrieved a group of uncharacterized proteins from the data base, including closely related WciG, a putative acetyl transferase involved in the synthesis of several *S. 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 Gal*f* and 6-OH of GalNAcα-1-PO_4^-) were based on ^{1}H and ^{13}C 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 Gal*f* and 30% at the 6-OH of GalNAcα-1-PO_4^-. Thus, the extent of *O*-acetylation appeared to vary somewhat with growth conditions.

To assess the role of *wefK*, we inserted this gene between *wzx* and *glf* of *S. gordonii* GC27 to obtain *S. gordonii* GC38, via the RPS^{-} transformant, *S. gordonii* GC29 (Table 1). In dot immunoblotting (Fig. 3*A*), strain GC38 exhibited reduced anti-type 1 immunoreactivity, compared 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. 3*A*) 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 ^{1}H and ^{13}C chemical shifts in NMR spectra of *S. gordonii* GC35 RPS, which expressed *wefI* 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. 3*A*) by inserting *wefK* between *wzx* 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* 38 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[a]	GalNAcα (A)	5.180, 95.81	4.233, 50.91	3.973, 68.00	4.078, 68.80	4.330, 70.44	4.01, 4.07, 65.18	2.087, 23.12
GC27	GalNAcα (A)	5.071, 95.13	4.224, 50.41	4.012, 68.08	4.063, 68.67	4.353, 70.33	3.997, 4.039, 65.05	2.054, 22.72
GC32	Galα (A)	5.127, 96.67	3.851, 69.13	3.963, 70.11	4.051, 69.72	4.344, 70.50	3.980, 4.024, 65.42	
GC51	Galα (A)	5.148, 97.18	3.903, 68.77	3.930, 69.89	4.068, 69.52	4.339, 70.37	3.88, 3.96, 64.78	
GC39	GalNAcα (A)	5.070, 95.50	4.225, 50.58	4.015, 68.35	4.066, 68.94	4.354, 70.70	3.996, 4.042, 65.42	2.057, 23.04
GC35	Galα (A)	5.127, 96.67	3.855, 69.13	3.963, 70.11	4.051, 69.72	4.348, 70.50	3.980, 4.026, 65.42	
Sg 38	Rhaβ (B)	4.925, 100.51	4.332, 73.05	3.727, 79.63	3.546, 71.75	3.476, 73.42	1.351, 17.70	
GC27	Rhaβ (B)	4.844, 101.13	4.207, 68.08	3.658, 78.33	3.428, 71.06	3.431, 72.72	1.339, 17.47	
GC32	Rhaβ (B)	4.888, 101.36	4.290, 68.35	3.695, 78.70	3.453, 71.28	3.458, 73.04	1.344, 17.77	
GC51	Rhaβ (B)	4.932, 101.76	4.371, 73.64	3.781, 79.11	3.525, 71.67	3.482, 73.43	1.355, 17.45	
GC39	Rhaβ (B)	4.845, 101.55	4.210, 68.35	3.659, 78.70	3.424, 71.28	3.434, 73.04	1.342, 17.77	
GC35	Rhaβ (B)	4.888, 101.36	4.295, 68.35	3.692, 78.70	3.455, 71.28	3.460, 73.04	1.344, 17.77	
Sg 38	Glcβ (C)	4.489, 103.53	3.341, 74.02	3.596, 76.83	3.604, 78.07	3.490, 75.60	3.783, 3.947, 61.80	
GC27	Glcβ (C)	4.512, 103.38	3.336, 73.79	3.650, 76.04	3.648, 77.16	3.533, 75.21	3.826, 3.943, 61.34	
GC32	Glcβ (C)	4.510, 103.70	3.346, 74.21	3.660, 76.36	3.656, 77.53	3.536, 75.58	3.839, 3.941, 61.71	
GC51	Glcβ (C)	4.497, 103.74	3.353, 74.04	3.612, 76.90	3.618, 78.15	3.489, 75.67	3.783, 3.958, 61.76	
GC39	Glcβ (C)	4.613, 102.92	3.322, 73.82	3.656, 76.17	3.649, 77.53	3.551, 75.58	3.847, 3.942, 61.71	
GC35	Glcβ (C)	4.615, 102.92	3.323, 73.82	3.665, 76.16	3.658, 77.53	3.538, 75.58	3.841, 3.941, 61.52	
Sg 38	Galfβ (D)	5.068, 108.66	4.070, 81.78	4.080, 77.53	3.989, 83.93	4.010, 70.51	3.741, 4.053, 72.08	
GC27	Galfβ (D)	5.061, 108.36	4.070, 81.51	4.070, 77.41	3.987, 83.61	4.014, 70.23	3.748, 4.053, 71.79	
GC32	Galfβ (D)	5.059, 108.78	4.071, 81.83	4.085, 77.73	3.990, 83.98	4.017, 70.50	3.751, 4.051, 72.26	
GC51	Galfβ (D)	5.067, 108.85	4.072, 81.87	4.086, 77.55	3.995, 84.01	4.010, 70.48	3.749, 4.061, 72.20	
GC39	Galfβ (D)	5.089, 108.58	4.247, 80.46	4.281, 85.34	4.132, 83.00	3.954, 71.28	3.681, 3.722, 63.67	
GC35	Galfβ (D)	5.088, 108.58	4.246, 80.46	4.280, 85.34	4.131, 83.00	3.953, 71.28	3.680, 3.716, 63.67	
Sg 38	GalNAcβ (E)	4.657, 104.01	3.956, 53.37	3.745, 71.63	3.944, 68.78	3.815, 74.58	3.746, 3.913, 68.03	2.045, 23.16
GC27	Galβ (E)	4.527, 105.09	3.526, 71.16	3.638, 73.06	3.929, 69.35	3.831, 74.28	3.760, 3.894, 67.88	
GC32	Galβ (E)	4.527, 105.46	3.524, 71.48	3.634, 73.43	3.924, 69.72	3.822, 74.60	3.753, 3.895, 68.16	
GC51	GalNAcβ (E)	4.645, 104.18	3.947, 53.34	3.751, 71.62	3.944, 68.80	3.829, 74.62	3.790, 3.906, 68.20	2.045, 23.16
GC39	Galβ (E)	4.533, 105.46	3.529, 71.48	3.639, 73.43	3.932, 69.52	3.834, 74.21	3.761, 3.893, 67.77	
GC35	Galβ (E)	4.529, 105.46	3.526, 71.48	3.636, 73.43	3.926, 69.52	3.826, 74.21	3.755, 3.897, 67.77	
Sg 38	Galα (F)	5.495, 96.58	3.895, 68.02	3.964, 79.68	4.242, 70.05	4.128, 72.38	3.73, 61.89	
GC27	GalNAcα (F)	5.486, 94.93	4.375, 49.23	4.078, 77.26	4.273, 69.15	4.163, 72.43	3.780, 3.758, 61.78	2.054, 22.72
GC32	GalNAcα (F)	5.489, 95.30	4.371, 49.60	4.085, 77.73	4.273, 69.52	4.158, 72.65	3.775, 3.761, 62.10	2.047, 23.04
GC51	Galα (F)	5.496, 96.73	3.895, 68.80	3.969, 79.68	4.247, 70.12	4.128, 72.40	3.73, 61.92	
GC39	GalNAcα (F)	5.487, 95.30	4.374, 49.41	4.074, 77.92	4.274, 69.33	4.159, 72.65	3.778, 3.756, 62.10	2.057, 23.04
GC35	GalNAcα (F)	5.486, 95.30	4.370, 49.41	4.070, 77.92	4.273, 69.33	4.156, 72.65	3.777, 3.760, 62.10	2.047, 23.04
Sg 38	Rhaα (G)	5.040, 100.92	4.041, 71.29	3.850, 71.10	3.449, 72.75	4.046, 69.47	1.265, 17.49	
GC51	Rhaα (G)	5.274, 101.58	4.084, 71.06	3.857, 71.00	3.441, 72.88	4.070, 69.46	1.278, 17.31	

[a] *Sg* 38 denotes type 2Gn RPS from *S. gordonii* 38 (9).

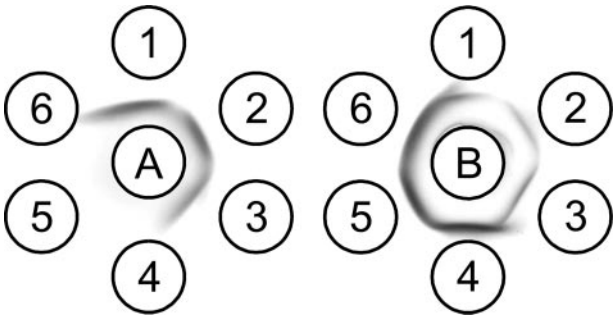


FIGURE 4. **Molecular basis of RPS serotype specificity revealed by immunodiffusion analysis of wild type and mutant polysaccharides.** Immunoprecipitation of rabbit antibody A against type 1G RPS-producing *S. oralis* MC2 with the RPS of *S. gordonii* GC27 (*circle 1*, type 1G), *S. gordonii* GC38 containing *wefK* (*circle 2*), or *S. gordonii* GC39 containing *wzy* of strain 10557 (*circle 3*) is correlated with the *wefA*-dependent presence of α-GalNAc in these polysaccharides. In contrast, strong immunoprecipitation of rabbit antibody B against type 3G RPS-producing *S. oralis* 10557 with the RPS of *S. gordonii* GC32 containing *wefI* (*circle 4*), *S. gordonii* GC37 (type 3G) containing *wefI*, *wefK*, and *wzy* of strain 10557 (*circle 5*), or *S. oralis* 10557 (*circle 6*, type 3G) is correlated with *wefI*-dependent presence of α-Gal in these polysaccharides.

GC37 RPS identified this polysaccharide as type 3G RPS and revealed partial *O*-acetylation at both expected positions (*i.e.* 5% to 10% at the 2-OH of Gal*f* and ~30% at the 6-OH of GalNAcα-1-PO_4^-). The extent of this modification was comparable with that seen in type 3G RPS isolated from strain 10557 cultured under the same conditions. As expected, the linkage and composition analyses of strain GC37 RPS (supplemental data) are in agreement with the proposed structure.

We also isolated *S. oralis* TC1 (Fig. 3*A*) by replacing *wefK* in *S. oralis* 10557 with the *ermAM* cassette by transformation of this strain in the presence of CSP. In contrast with NMR spectra of strain 10557 RPS, the NMR spectra of strain TC1 RPS showed no evidence of *O*-acetylation, thereby further associating *wefK* with this modification. Immunostaining of strains TC1 and 10557 in dot immunoblotting, like that seen with strains GC35 and GC37, was only noted following incubation of these strains with anti-type 3G RPS-specific IgG (Fig. 3*A*). Thus, *O*-acetylation, which is well known for its effect on the antigenicity of bacterial polysaccharides (14, 26, 27), did not appear to be a critical determinant of RPS immunoreactivity.

Identification of wefA and wefI as Molecular Determinants of RPS Serotype Specificity—Serotype-specific reactions of different strains in dot immunoblotting (Fig. 3A) were only observed on blots incubated with relatively low concentrations of primary antibody (*i.e.* 12 ng of IgG/ml). To verify the essential results of these determinations, we performed conventional immunodiffusion experiments (Fig. 4) with the purified polysaccharides of selected strains and high titer rabbit antistreptococcal serum prepared against type 1G RPS-producing *S. oralis* MC2 (18) or type 3G RPS-producing *S. oralis* 10557. Antiserum against type 1G RPS (Fig. 4, *left panel*) gave a reaction of identity with type 1G RPS of *S. gordonii* GC27 (well 1), RPS from *wefK*-containing *S. gordonii* GC38 (well 2) and RPS from *S. gordonii* GC39, which expressed *wzy* of *S. oralis* 10557 (well 3). In contrast, RPS from *wefI*-containing *S. gordonii* GC32 (well 4) was unreactive, thereby indicating that the presence of α-Gal in place of α-GalNAc abolished the reaction of serotype 1-specific antibodies. In a parallel experiment performed with antiserum against type 3G RPS (Fig. 4, *right 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 wefJ 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.* WefJ in Fig. 2). To examine this possibility, we tested *wefJ* 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 *wefJ* genetically linked to the *ermAM* cassette. The presence of *ermAM* did not have a polar effect on the expression of downstream genes. The resulting RPS^+ transformant, *S. oralis* MC11 reacted weakly with anti-type 1Gn/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 L-Rha branches. This finding suggested that the action of WefJ was limited to the relatively small amount of linear acceptor that remained available in the presence of WefB. Finally, we constructed *S. oralis* MC12 (Fig. 5) by inserting *wefJ* (linked to the nonpolar *ermAM* 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 WefJ 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-

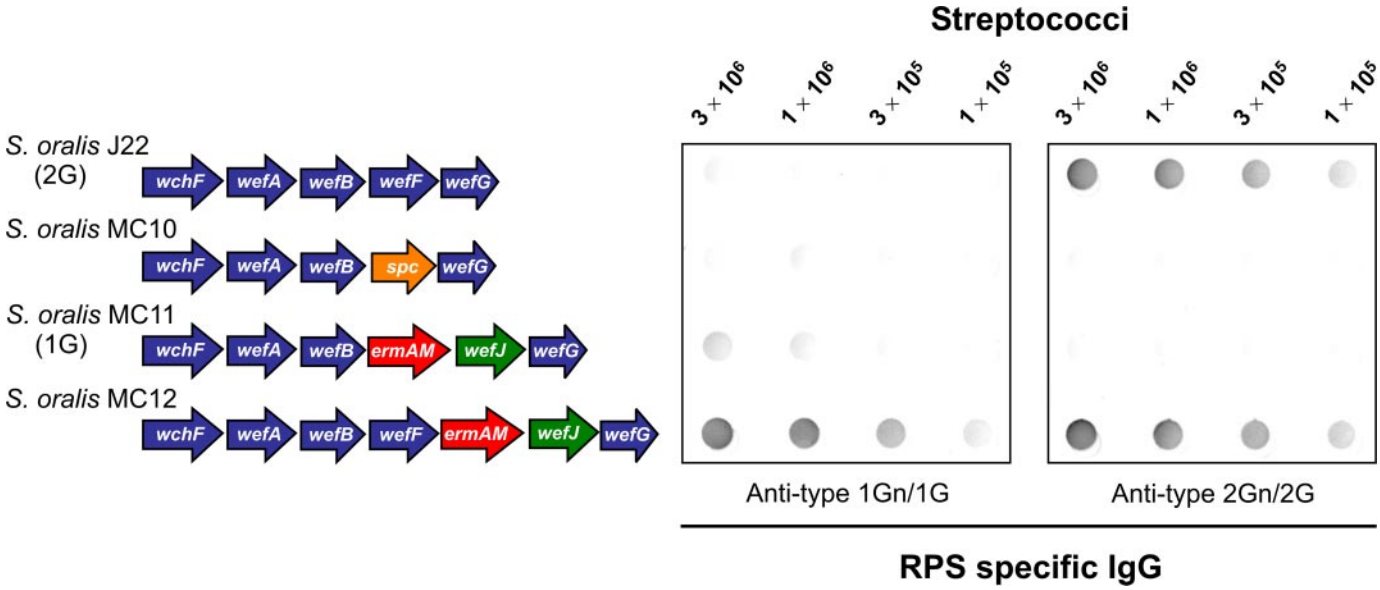


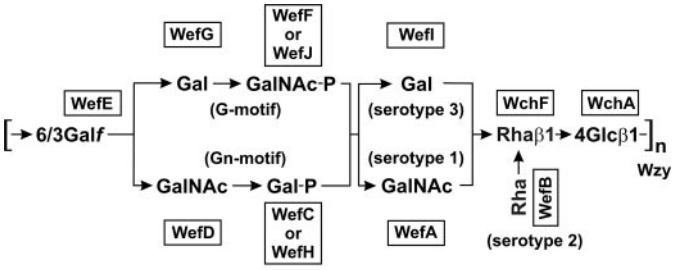
FIGURE 5. **Dot immunoblots of wild type and mutant streptococci with anti-type 1Gn/1G and anti-type 2Gn/2G RPS-specific antibodies showing a difference in the acceptor specificities of the GalNAc-1-phosphotransferases encoded by *wefF* of *S. oralis* J22 and allelic *wefJ* of *S. oralis* 10557.** ORF 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 *wefJ* (linked to *ermAM*), produced a relatively small amount of type 1G RPS, which was identified by HSQC spectra of the isolated polysaccharide.

tical to the corresponding region in strain SK23. Greater than 96% nucleotide sequence identity also was noted between the sequences of *wefG* or intact *wzy* in these strains and the same genes in strains SK120, 4477, or H127. However, the regions between these genes in the latter three strains differed both in length (*i.e.* 797, 767, and 1222 bp, respectively) and in the size of their truncated *wzy*-like ORFs (*i.e.* 116, 82, and 247 encoded amino acid residues, respectively). The values for the percentages of amino acid sequence identity were 57% for the first 60 amino acid residues of the truncated *wzy*-like ORFs in the five strains that produced type 3G RPS, 69% for the corresponding regions of Wzy in *S. oralis* 34, *S. gordonii* 38, and *S. oralis* J22, and 72% for the consensus sequences of these two groups. Importantly, strains 34, 38, and J22 Wzy were the only bacterial sequences retrieved from the existing data base by the consensus sequence of truncated Wzy. Thus, the truncated ORFs identified in each of five type 3G RPS-producing strains appeared to be remnants of the same recombinational event, namely the replacement of one *wzy* with another, resulting in a change in the linkage between repeating units from β1–6 in an ancestral polysaccharide to β1–3 in type 3G RPS.

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 *wefE*) account for the common presence of Rhaβ1–4Glc at one end and Gal*f* 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, WefJ, WefC, and WefH) that add Gal-1-PO_4^- or GalNAc-1-PO_4^- to the branched or linear acceptors associated with different serotypes. The other group encodes WefG and WefD, which catalyze the subsequent β1–3 transfer of Gal or GalNAc, respectively, completing the recognition motifs in these polysaccharides. The WefE-dependent transfer of Gal*f* 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 Gal*f* 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



Protein	Donor	Linkage	Acceptor	Strain
WefI	Gal	α1-3	→ Rhaβ1-4Glc-	10557, GC32
WefA	GalNAc	α1-3		38
WefB	Rha	α1-2	GalNAcα1-3Rhaβ1-4Glc-	38
			Galα1-3Rhaβ1-4Glc-	GC51
WefF	GalNAcα1-PO_4^-	α1-6	GalNAcα1-3Rhaβ1-4Glc- (Rhaα1-2)	J22, MC12, GC16
			GalNAcα1-3Rhaβ1-4Glc-	MC2, GC27
			Galα1-3Rhaβ1-4Glc-	GC32
WefJ	GalNAcα1-PO_4^-	α1-6	Galα1-3Rhaβ1-4Glc-	10557
			GalNAcα1-3Rhaβ1-4Glc-	MC11, MC12
WefC	Galα1-PO_4^-	α1-6	GalNAcα1-3Rhaβ1-4Glc- (Rhaα1-2)	38
			Galα1-3Rhaβ1-4Glc- (Rhaα1-2)	GC51
			GalNAcα1-3Rhaβ1-4Glc-	GC12
WefH	Galα1-PO_4^-	α1-6	GalNAcα1-3Rhaβ1-4Glc-	34, GC48
WefG	Gal	β1-3	GalNAcα1-PO_4^--6GalNAc-	J22
			Galα1-PO_4^--6GalNAc-	GC19
WefD	GalNAc	β1-3	Galα1-PO_4^--6GalNAc-	38
			GalNAcα1-PO_4^--6GalNAc-	GC17
WefE	Galf	β1-3	GalNAcβ1-3Galα1-PO_4^--	38
			Galβ1-3GalNAcα1-PO_4^--	GC16
			GalNAcα1-PO_4^--6GalNAc-	GC28
			Galα1-PO_4^--6GalNAc-	GC14

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.

two positions of the strain 10557 repeat. Additional findings demonstrate the relaxed acceptor specificities of allelic WefF, WefJ, and WefC for Gal- and GalNAc-containing structures by the apparent ability of these encoded proteins to act on the product of either WefI or WefA. They also distinguish the allelic GalNAc-1-phosphotransferases, WefF and WefJ, by their ability to act on the branched acceptor formed by the action of WefB (Fig. 5).

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 digests 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. 3*B*) 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* 38 (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 Wzy-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

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 Rhaα1–2Rha 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.

Acknowledgments—We thank Shuntaro Ito and Kazushi Kunimatsu for contributions and Dwayne Lunsford for helpful review of the manuscript. We acknowledge Biswa Choudhury of the Complex Carbohydrate Research Center at the University of Georgia for performing the composition and linkage analyses and Russell Carlson for advice on interpretation.

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