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Molecular Basis of L-Rhamnose Branch Formation in Streptococcal Coaggregation Receptor Polysaccharides†

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The presence of L-rhamnose (Rha) branches in the coaggregation receptor polysaccharides (RPS) of *Streptococcus gordonii* 38 and *Streptococcus oralis* J22 was eliminated by replacement of *wefB* with *ermAM* in these strains. The expression of this gene in *S. oralis* 34 did not, however, result in the addition of Rha branches to the linear RPS of this strain, which is identical to that produced by the *wefB*-deficient mutant of *S. gordonii* 38. This paradoxical finding was explained by a subtle difference in acceptor specificity of the galactose-1-phosphotransferases encoded by downstream *wefC* in *S. gordonii* 38 and *wefH* in *S. oralis* 34. These genes were distinguished by the unique ability of WefC to act on the branched acceptor formed by the action of WefB.

Interactions of viridans group streptococci with other oral species contribute to early biofilm formation during microbial colonization of the human tooth surface (10, 15, 16). These interactions generally depend on the binding of lectin-like adhesins, such as those associated with the type 2 fimbriae of *Actinomyces naeslundii* (7), to different streptococcal coaggregation receptor polysaccharides (RPS), including type 1Gn of *Streptococcus oralis* 34 (3, 13), type 2Gn of *Streptococcus gordonii* 38 (17), and type 2G of *S. oralis* J22 (2) (Fig. 1). Adhesin-mediated recognition of these polysaccharides depends on the presence of host-like features, either GalNAcβ1-3Gal (Gn) or Galβ1-3GalNAc (G), in the repeating units of these molecules (5). In contrast, the reactions of these polysaccharides as antigens reflect other features such as the L-rhamnose (Rha) branch of type 2Gn RPS, which forms the major epitope of this polysaccharide (17) at the expense of immunodominant α-GalNAc in type 1Gn RPS (14).

The structural difference between type 2Gn RPS of *S. gordonii* 38 and type 2G RPS of *S. oralis* J22 (Fig. 1) depends on two of the seven genes for glycosyltransferases in the RPS gene cluster of each strain (Fig. 2), *wefC* and *wefD* in strain 38 versus *wefF* and *wefG* in strain J22 (21). Three of the remaining genes (i.e., *wchA*, *wchF*, and *wefE*) for glycosyltransferases in each RPS gene cluster have also been associated with individual biosynthetic steps (Fig. 3), leaving *wefA* and *wefB* as likely candidates for the transfer of α-GalNAc and α-Rha to Rhaβ1-4Glc (20). The specific roles of *wefA* and *wefB* in synthesis of the resulting branched structure is, however, unclear, as neither gene has a well-studied homologue in the database.

Type 2Gn RPS of *S. gordonii* 38 is identical to type 1Gn RPS of *S. oralis* 34 except for the presence of Rha branches in the former polysaccharide (Fig. 1). Therefore, to gain insight into the role of *wefA* or *wefB* in branch formation, we identified and sequenced the *S. oralis* 34 RPS gene cluster and flanking regions (GenBank accession no. AB181234), following methods essentially identical to those previously described in studies of *S. oralis* J22 (21). The RPS gene cluster of *S. oralis* 34, which was PCR amplified from genomic DNA of this strain, more closely resembled the RPS gene cluster of *S. oralis* J22 than that of *S. gordonii* 38 (Fig. 2), both in terms of its overall location between the genes for dextranase (*dexB*) and an oligopeptide-binding protein (*aliA*) and in terms of its association with the four *rml* genes for dTDP–L-Rha biosynthesis. Of greater interest was the presence of six rather than seven genes for putative glycosyltransferases in the RPS gene cluster of *S. oralis* 34, including *wefA* but not *wefB* (Fig. 2). Thus, *wefA* may be associated with the transfer of α-GalNAc in types 1Gn, 2Gn, and 2G RPS (Fig. 1) and *wefB* with synthesis of the Rha branches in type 2Gn and 2G RPS.

To test these hypotheses, we replaced various specific genes in *S. gordonii* 38, *S. oralis* 34, or *S. mitis* J22 as previously described (21) by transforming these bacteria with DNA constructs containing the *ermAM* cassette (12) flanked by appropriate 0.5- to 1-kb gene-targeting sequences. The constructs used as transforming DNA were prepared by overlap extension PCR (9, 11). The location of the cassette in the resulting erythromycin-resistant transformants (Table 1) was verified by amplification of specific PCR products across the upstream and downstream boundaries of the *ermAM* insertion (results not shown). Cell surface RPS production by wild-type and mutant strains was characterized and compared by the binding of different RPS-specific probes to decreasing numbers of bacteria immobilized on nitrocellulose membranes (21). The probes utilized included anti-type 1 RPS specific immunoglobulin G (IgG) from antiserum against *S. oralis* 34, which was purified by elution from coupled type 1Gn RPS (16), anti-type 2 RPS specific IgG from antiserum R103 against *S. gordonii* 38 (6), which was purified by elution (20) from coupled type 2G RPS of *S. oralis* J22, and biotin-labeled *A. naeslundii* 12104

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Type 2Gn RPS (*S. gordonii* 38):

$$[\rightarrow 6Gal\mathit{f}\beta1\rightarrow 6GalNAc\beta1\rightarrow 3Gal\alpha1\text{-}PO_4^-\rightarrow 6GalNAc\alpha1\rightarrow 3Rha\beta1\rightarrow 4Glc\beta1\rightarrow]_n$$
$$Rha\alpha1^{\nearrow 2}$$

Type 1Gn RPS (*S. oralis* 34):

$$[\rightarrow 6Gal\mathit{f}\beta1\rightarrow 6GalNAc\beta1\rightarrow 3Gal\alpha1\text{-}PO_4^-\rightarrow 6GalNAc\alpha1\rightarrow 3Rha\beta1\rightarrow 4Glc\beta1\rightarrow]_n$$

Type 2G RPS (*S. oralis* J22):

$$[\rightarrow 6Gal\mathit{f}\beta1\rightarrow 6Gal\beta1\rightarrow 3GalNAc\alpha1\text{-}PO_4^-\rightarrow 6GalNAc\alpha1\rightarrow 3Rha\beta1\rightarrow 4Glc\beta1\rightarrow]_n$$
$$Rha\alpha1^{\nearrow 2}$$

Type 1G RPS (*S. oralis* MC2):

$$[\rightarrow 6Gal\mathit{f}\beta1\rightarrow 6Gal\beta1\rightarrow 3GalNAc\alpha1\text{-}PO_4^-\rightarrow 6GalNAc\alpha1\rightarrow 3Rha\beta1\rightarrow 4Glc\beta1\rightarrow]_n$$

FIG. 1. Structural types of RPS produced by different streptococci in the present study.

(4, 18), which binds both Gn and G types of streptococcal cell surface RPS (5, 21).

Cell surface RPS production was abolished by *ermAM* replacement of either *wchA* in *S. oralis* 34 or *wchF* or *wefA* in *S. gordonii* 38, the genes for the first three predicted steps in RPS biosynthesis (Fig. 3). Thus, the resulting mutants, *S. oralis* OC1, *S. gordonii* GC10, or *S. gordonii* GC21, respectively, failed to bind either RPS-specific antibody or *A. naeslundii* (Fig. 4). In contrast, *ermAM* replacement of *wefB* in *S. gordonii* 38 or *S. oralis* J22 altered the immunoreactivity of these bacteria without affecting the binding of *A. naeslundii* (Fig. 4). The parental strains 38 and J22 bound anti-type 2 but not anti-type 1 RPS specific IgG, whereas the *wefB* mutants (i.e., *S. gordonii* GC12 and *S. oralis* MC2, respectively) exhibited the opposite pattern of reactivity. In further studies, the ability of *wefB* to complement type 2Gn RPS production in *trans* was demonstrated following the cloning of this gene in pYY101 (Table 1) and transformation of an erythromycin-sensitive *wefB* deletion mutant of *S. gordonii* 38 with the resulting plasmid (results not shown).

To establish the structural basis for the switch in antigenicity associated with the deletion of *wefB*, we solubilized and purified the RPS of *S. gordonii* GC12 and *S. oralis* MC2 and recorded complete sets of homonuclear and heteronuclear (^{1}H and ^{13}C) NMR spectra at 500 MHz ^{1}H frequency following previously described methods (1–3, 6, 21). The peaks in a heteronuclear single-quantum coherence (HSQC) spectrum of *S. gordonii* GC12 RPS (data not shown) corresponded exactly with the ^{1}H and ^{13}C resonances previously assigned for type 1Gn RPS of *S. oralis* 34 (3). Since the structure of *S. oralis* MC2 RPS was expected to be novel, a complete assignment of the ^{1}H and ^{13}C resonances was undertaken using standard homonuclear methods (correlation spectroscopy [COSY], total correlation spectroscopy [TOCSY], and nuclear Overhauser spectroscopy [NOESY]) and heteronuclear methods (HSQC and heteronuclear multiple bond correlation [HMBC]). The resulting assignments are reported in Fig. S1 and Table S1 in the supplemental material. Linkages between residues were determined using HMBC and NOESY as indicated in Table S2

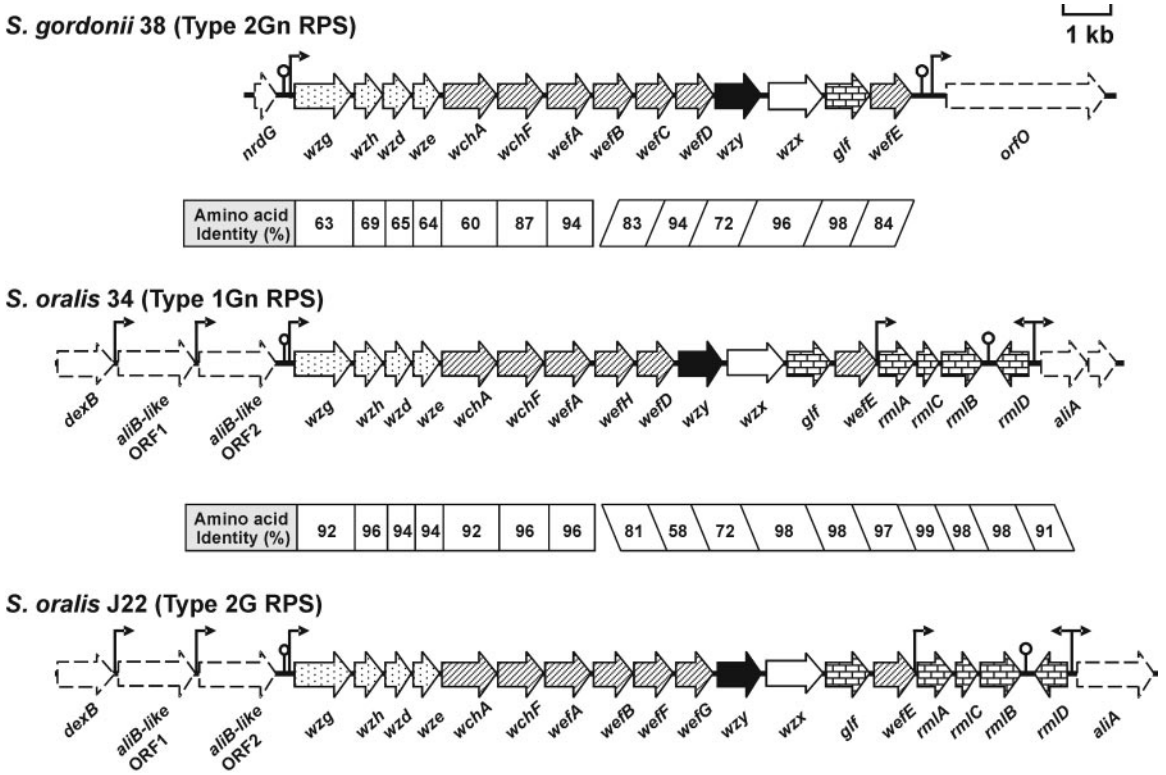

FIG. 2. ORF diagrams of RPS gene clusters indicating the homology between strains and the predicted roles of different genes in RPS biosynthesis. Each cluster contains four common regulatory genes (stippled arrows), six or seven genes for glycosyl or glycosyl-1-phosphotransferases (hatched arrows), and additional genes for a polysaccharide polymerase (solid arrows), a repeat unit transporter (open arrows), and enzymes for nucleotide sugar biosynthesis (brick-pattern arrows). Flanking genes (arrows with dashed outlines) are also identified, as are the positions of putative promoters (bent arrows) and rho-independent terminators (lollipops).

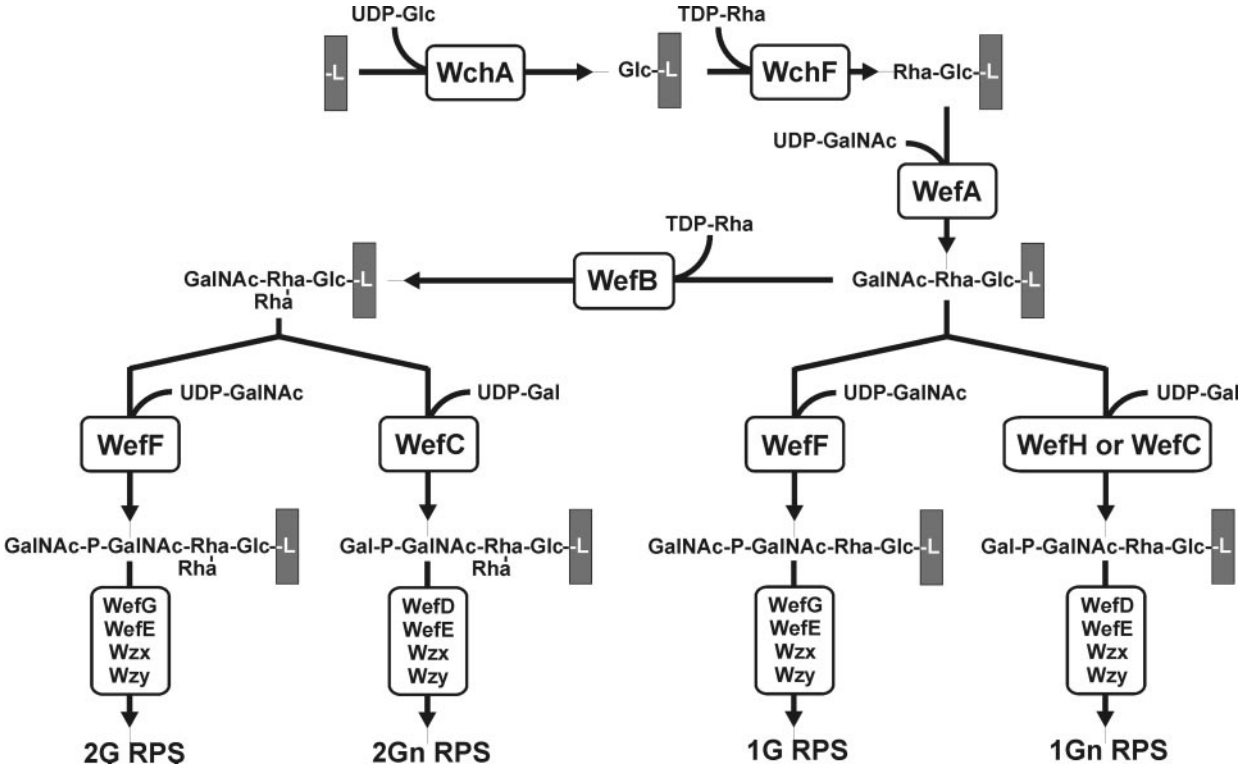

FIG. 3. Proposed pathway for biosynthesis of types 2G, 2Gn, 1G, and 1Gn RPS beginning with the WchA-catalyzed transfer of Glc-1-PO_4^- to carrier lipid (L). The present findings define the roles of WefA and WefB in RPS biosynthesis and reveal a difference in the specificities of WefC and WefH for branched versus linear acceptors.

in the supplemental material. These data identify the *S. oralis* MC2 polysaccharide as type 1G RPS (Fig. 1), a new structural type. In immunodiffusion experiments, this polysaccharide and type 1Gn RPS of *S. oralis* 34 reacted identically with different rabbit antisera against either *S. oralis* 34 or *S. oralis* MC2 (results not shown). Thus, the structural and corresponding antigenic difference between type 2Gn or 2G RPS and type 1Gn or 1G RPS clearly depends on the glycosyltransferase encoded by *wefB*.

The finding that *wefB* directs the synthesis of Rha branches

TABLE 1. Streptococci and plasmids used in this study

Strain or plasmid	Description	Reference
Strains[a]		
S. oralis 34	Wild-type strain (type 1Gn RPS)	3
S. oralis OC1	*S. oralis* 34 containing *ermAM* in place of *wchA*	This study
S. oralis OC5	*S. oralis* 34 containing *wefB* of *S. gordonii* 38 and *ermAM* between *wefA* and *wefH*	This study
S. gordonii 38	Wild-type strain (type 2Gn RPS)	17
S. gordonii GC10	*S. gordonii* 38 containing *ermAM* in place of *wchF*	This study
S. gordonii GC12	*S. gordonii* 38 containing *ermAM* in place of *wefB*	This study
S. gordonii GC13	*S. gordonii* 38 containing *ermAM* in place of *wefC*	21
S. gordonii GC20	*S. gordonii* 38 containing *ermAM* in place of *wzy*	This study
S. gordonii GC21	*S. gordonii* 38 containing *ermAM* in place of *wefA*	This study
S. gordonii GC22	*S. gordonii* 38 containing *ermAM* in place of *wzx*	This study
S. gordonii GC45	*S. gordonii* GC20 containing *wzy* of *S. oralis* 34 in place of *ermAM*	This study
S. gordonii GC48	*S. gordonii* GC13 containing *wefH* of *S. oralis* 34 in place of *ermAM*	This study
S. gordonii GC49	*S. gordonii* GC22 containing *wzx* of *S. oralis* 34 in place of *ermAM*	This study
S. gordonii GC50	*S. gordonii* GC21 containing *wefA* of *S. oralis* 34 in place of *ermAM*	This study
S. oralis J22	Wild-type strain (type 2G RPS)	2
S. oralis MC2	*S. oralis* J22 containing *ermAM* in place of *wefB*	This study
Plasmids		
pYY101	pCM18 lacking *gfp*; confers Em^r	8; this study
pYY110	pYY101 containing *wefH* from *S. oralis* 34	This study

[a] Streptococci were grown in Todd-Hewitt broth (Difco) containing erythromycin (10 μg/ml) as needed for the maintenance of *ermAM*.

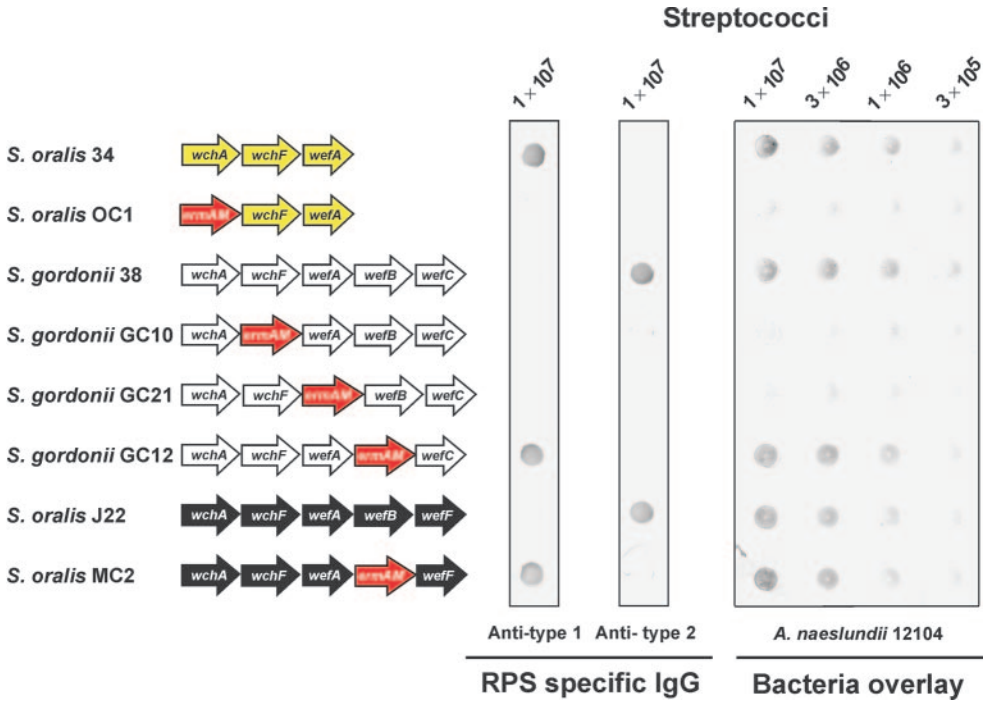

FIG. 4. RPS production by wild-type and mutant streptococci detected by dot immunoblotting with RPS-specific rabbit IgG or binding of biotin-labeled *A. naeslundii* 12104. Nitrocellulose membranes were spotted with streptococci, incubated with RPS-specific rabbit IgG or biotin-labeled *A. naeslundii*, washed, and developed with goat anti-rabbit IgG or horseradish peroxidase-conjugated avidin, followed by a substrate to detect bound IgG or *A. naeslundii*, respectively. Partial ORF diagrams of wild-type and mutant streptococci indicate the presence of genes from *S. oralis* 34 (yellow arrows), *S. gordonii* 38 (white arrows), *S. oralis* J22 (black arrows), or *ermAM* (red arrows).

in type 2Gn and 2G RPS leaves *wefA* for the transfer of α-GalNAc to Rhaβ1-4Glc in these polysaccharides. Experimental evidence supporting this role has been obtained in recent molecular studies of *S. oralis* 10557 type 3G RPS, which contains α-Gal rather than α-GalNAc as in type 2Gn RPS (Y. Yoshida et al., manuscript in preparation). While further studies are needed to verify the order of addition, we suspect that the WefA-catalyzed formation of GalNAcα1-3Rhaβ in linear type 1Gn RPS precedes the WefB-catalyzed addition of Rhaα to Rhaβ (Fig. 3), as the alternative would require that WefA utilize subterminal Rha as an acceptor in biosynthesis of type 2Gn and 2G RPS and terminal Rha as an acceptor in biosynthesis of type 1Gn RPS.

Based on the above findings, we expected that the expression of *wefB* in *S. oralis* 34 would switch RPS production from type 1Gn to type 2Gn. To examine this possibility, we isolated a *wefB*-inserted transformant of *S. oralis* 34 (i.e., *S. oralis* OC5) following transformation of this wild-type strain with *wefB* linked to the selectable *ermAM* cassette. Transforming DNA was prepared by two rounds of overlap extension PCR, the first to link *wefB* to *ermAM* and the second to link the resulting *wefB-ermAM* fragment to targeting sequences for insertion between *wefA* of *S. oralis* 34 and the downstream gene, which is identified below as *wefH*. The precise insertion of the *wefB-ermAM* fragment between *wefA* and *wefH* in *S. oralis* 34 was confirmed by DNA sequencing of this region in *S. oralis* OC5. Surprisingly, this mutant, which expresses *wefB* from the promoter at the 5′ end of the RPS gene cluster and downstream genes for RPS biosynthesis from the promoter in the *ermAM* cassette, as well a similar mutant in which the order of *wefB* and *ermAM* was reversed (results not shown), reacted with anti-type 1 but not anti-type 2 RPS specific IgG in dot immunoblotting (Fig. 5). Consistent with these reactions, the resonances in the HSQC NMR spectrum of the RPS purified from *S. oralis* OC5 corresponded exactly to the ^{1}H and ^{13}C assignment for *S. oralis* 34 type 1Gn RPS (3), with no indication (i.e., less than 5% [data not shown]) of Rha branches.

The above findings raise the possibility that the synthesis of Rha branches in *S. gordonii* 38 RPS depends on more than simply the expression of *wefB* in this strain. To explore this possibility, we replaced *wefA*, *wefC*, *wzy*, or *wzx* in *S. gordonii* 38 with the corresponding gene of *S. oralis* 34 by following a previously described strategy (21). The gene of interest in *S. gordonii* 38 was initially replaced by the *ermAM* cassette as described above to obtain *S. gordonii* GC21, GC13, GC20, and GC22, respectively (Table 1), which grew on erythromycin and were nonreactive with RPS-specific IgG. These strains were then transformed with overlap extension PCR products that contained individual genes of *S. oralis* 34 (i.e., *wefA*, *wefH*, *wzy*, or *wzx*, respectively) flanked by targeting sequences for the *S. gordonii* 38 genes present on either side of *ermAM* cassette. Replacement of the *ermAM* cassette with a complementary gene from *S. oralis* 34 restored cell surface RPS production, which was detected by colony immunoblotting (21), resulting in the isolation of *S. gordonii* GC50, GC48, GC45, and GC49, respectively (Table 1). The reactions of mutant *S. gordonii* GC50, GC45, and GC49 with anti-type 2 but not anti-type 1 RPS specific IgG were indistinguishable from those of wild-

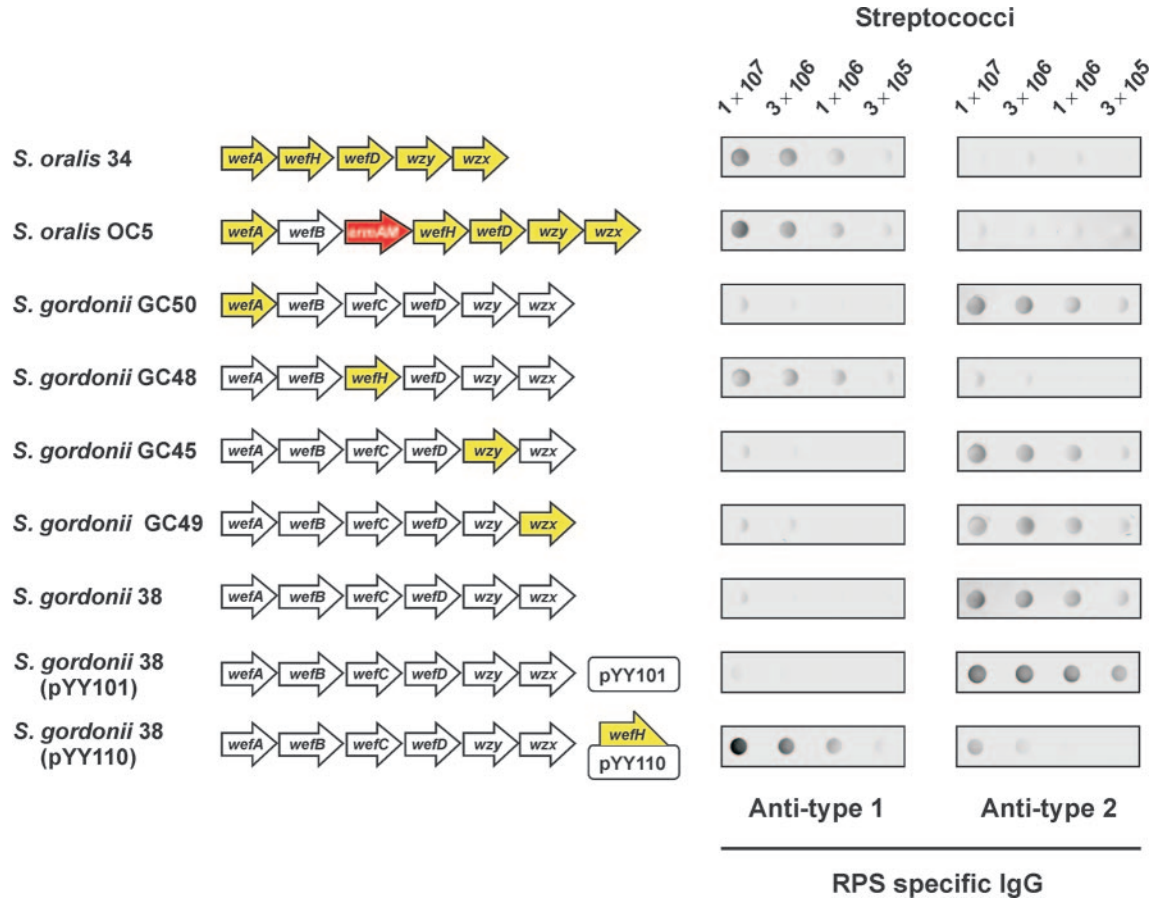

FIG. 5. RPS production by wild-type and mutant streptococci detected by dot immunoblotting with anti-type 1 or anti-type 2 RPS specific rabbit IgG. Nitrocellulose membranes were spotted with decreasing numbers of streptococci, incubated with RPS-specific rabbit IgG, washed, and developed with goat anti-rabbit IgG followed by a substrate to detect bound IgG. Partial ORF diagrams of wild type and mutant streptococci strains indicate the presence of genes from *S. oralis* 34 (yellow arrows), *S. gordonii* 38 (white arrows), or *ermAM* (red arrows).

type *S. gordonii* 38 (Fig. 5), thereby indicating that *wefA*, *wzy*, and *wzx* of *S. oralis* 34 all support type 2Gn RPS production.

In contrast, *S. gordonii* GC48, obtained by replacement of *wefC* in *S. gordonii* 38 with the corresponding gene from *S. oralis* 34, reacted with anti-type 1 but not anti-type 2 RPS specific IgG (Fig. 5), thereby associating the production of type 1Gn RPS with the presence of a unique gene (i.e., *wefH*) in the latter strain. The antigenic identification of type 1Gn RPS on *S. gordonii* GC48 was confirmed by the HSQC NMR spectrum of the RPS purified from this strain (results not shown). In support of these findings, the plasmid-based expression of *wefH* in *S. gordonii* 38 altered the RPS-specific immunoreactivity of this strain. Whereas *S. gordonii* 38 harboring control plasmid pYY101 reacted with anti-type 2 but not anti-type 1 RPS specific IgG, strain 38 harboring pYY110, which expresses *wefH*, reacted strongly with anti-type 1 and weakly with anti-type 2 RPS specific IgG (Fig. 5). Thus, the WefH-mediated transfer of Galαl-PO_4^- to GalNAcα (Fig. 3) prevents WefB from acting, presumably by eliminating the acceptor of this enzyme. This effect may, in turn, limit the accumulation of lipid-linked branched tetrasaccharide in *S. gordonii* GC48, *S. gordonii* 38 (pYY110), and *S. oralis* OC5 by the action of WefB in these strains. Conversely, the branched acceptor formed by the WefB-catalyzed transfer of Rhaα to subterminal Rhaβ may not be recognized by WefH, a possibility consistent with the known ability of the Rha branch to block the binding of anti-type 1 antibody (14, 17) or the *Codium fragile* lectin (5) to adjacent GalNAcα of type 2Gn RPS.

WefC contributes to the synthesis of linear type 1Gn RPS in *wefB*-deficient *S. gordonii* GC12 (Fig. 4) and of branched-type 2Gn RPS in *wefB*-containing *S. gordonii* 38 (Fig. 3). The preference of WefC for the branched acceptor structure synthesized by strain 38 can, however, be inferred from the NMR spectra of type 2Gn RPS (17), which indicate the absence of detectable (<5%) linear hexasaccharide repeats in this polysaccharide. Likewise, WefF of *S. oralis* J22 utilizes a branched acceptor in synthesis of type 2G RPS and a linear acceptor in synthesis of type 1G RPS by *wefB*-deficient *S. oralis* MC2 (Fig. 3 and 4). The identification and structural characterization of type 1G RPS in the present investigation should facilitate the identification of wild-type streptococci that produce this polysaccharide. Once they are identified, it will be of interest to determine whether these bacteria utilize WefF to transfer GalNAcαl-PO_4^- to GalNAcα or a closely related enzyme that only acts on the linear acceptor formed in the absence of WefB (Fig. 3).

The proteins encoded by *wefH* of *S. oralis* 34, *wefC* of *S. gordonii* 38, and *wefF* of *S. oralis* J22 have pivotal roles in RPS biosynthesis, linking the recognition and antigenic regions of these polysaccharides. This biological role is a direct reflection of the donor specificities of these enzymes for Galα1-PO_4^- or GalNAcα1-PO_4^- and their acceptor specificities for linear or branched structures. In view of the relatively high homology that exists between these proteins (Fig. 2), their ability to discriminate between different substrates is likely to depend on minor differences in amino acid sequence. The identification of such sequences and their association with the synthesis of different types of RPS would contribute to the further characterization of these proteins as members of a recently recognized group of glycosyl-1-phosphotransferases (19) and of the corresponding genes as genetic markers of oral biofilm development.

Nucleotide sequence accession number. The *S. oralis* 34 RPS gene cluster and flanking regions have been deposited in GenBank under accession no. AB181234.

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