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Carbohydrate engineering of the recognition motifs in streptococcal co-aggregation receptor polysaccharides

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Summary

The cell wall polysaccharides of certain oral streptococci function as receptors for the lectin-like surface adhesins on other members of the oral biofilm community. Recognition of these receptor polysaccharides (RPS) depends on the presence of a host-like motif, either GalNAcβ1-3Gal (Gn) or Galβ1-3GalNAc (G), within the oligosaccharide repeating units of different RPS structural types. Type 2Gn RPS of *Streptococcus gordonii* 38 and type 2G RPS of *Streptococcus oralis* J22 are composed of heptasaccharide repeats that are identical except for their host-like motifs. In the current investigation, the genes for the glycosyltransferases that synthesize these motifs were identified by high-resolution nuclear magnetic resonance (NMR) analysis of genetically altered polysaccharides. RPS production was switched from type 2Gn to 2G by replacing *wefC* and *wefD* in the type 2Gn gene cluster of *S. gordonii* 38 with *wefF* and *wefG* from the type 2G cluster of *S. oralis* J22. Disruption of either *wefC* or *wefF* abolished cell surface RPS production. In contrast, disruption of *wefD* in the type 2Gn cluster or *wefG* in the type 2G cluster eliminated β-GalNAc from the Gn motif or β-Gal from the G motif, resulting in mutant polysaccharides with hexa- rather than heptasaccharide subunits. The mutant polysaccharides reacted like wild-type RPS with rabbit antibodies against type 2Gn or 2G RPS but were inactive as co-aggregation receptors. Additional mutant polysaccharides with GalNAcβ1-3GalNAc or Galβ1-3Gal recognition motifs were engineered by replacing *wefC* in the type 2Gn cluster with *wefF* or *wefF* in the type 2G cluster with *wefC* respectively. The reactions of these genetically modified polysaccharides as antigens and receptors provide further insight into the structural basis of RPS function.

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Introduction

Viridans group streptococci are primary colonizers of the human tooth surface (Nyvad and Kilian, 1987). These bacteria attach to salivary components that coat the mineral surface and form a relatively simple biofilm community through growth and interactions with other bacteria. These interactions generally result from binding of galactose (Gal)- and *N*-acetylgalactosamine (GalNAc)-reactive adhesins on species such as *Actinomyces naeslundii* to surface receptors on the streptococci that initiate colonization (Hsu *et al*., 1994; Palmer *et al*., 2003). Six different streptococcal receptor polysaccharides (RPS) have been identified from over 20 *Streptococcus sanguis*, *S. gordonii*, *S. oralis* and *S. mitis* strains that co-aggregate with *A. naeslundii* (Cisar *et al*., 1997). Each structural type of RPS is composed of a distinct hexa- or heptasaccharide repeating unit and each repeating unit contains a host-like motif, either GalNAcβ1-3Gal (Gn) or Galβ1-3GalNAc (G) (Cisar *et al*., 1995). Four structural types of RPS contain Gn motifs (i.e. RPS types 1Gn, 2Gn, 4Gn and 5Gn) and two types contain G motifs (i.e. RPS types 2G and 3G). These motifs are underlined in the closely related structures of *S. gordonii* 38 type 2Gn RPS (Reddy *et al*., 1994) and *S. oralis* J22 type 2G RPS (Abeygunawardana *et al*., 1990) given below.

Type 2Gn RPS:

$$[\text{-6Gal}f\beta\text{1-6}\underline{\text{GalNAc}\beta\text{1-3Gal}}\alpha\text{1-PO}_4^{-}\text{-6GalNAc}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$$
$$\text{Rha}\alpha\text{1-2}\lrcorner$$

Type 2G RPS:

$$[\text{-6Gal}f\beta\text{1-6}\underline{\text{Gal}\beta\text{1-3GalNAc}}\alpha\text{1-PO}_4^{-}\text{-6GalNAc}\alpha\text{1-3Rha}\beta\text{1-4Glc}\beta\text{1-}]_n$$
$$\text{Rha}\alpha\text{1-2}\lrcorner$$

Whereas both Gn- and G-types of RPS are receptors of *A. naeslundii* type 2 fimbriae, only Gn-types of RPS are recognized by the GalNAc-specific adhesins present on non-RPS producing strains of *S. sanguis* and *S. gordonii* (Cisar *et al*., 1997). Recognition of the host-like features in these linear polysaccharides may depend on other features of these molecules such as the flexible β1-6 linkage from adjacent Gal*f* as well as the adjacent anionic phosphodiester group (McIntire *et al*., 1988; Xu and Bush, 1996). The host-like features of these polysaccharides,

although critical for interbacterial adhesion, contribute little to antigenicity. Instead, major immunological epitopes include features such as the common Rha branch region of types 2Gn and 2G RPS (Reddy *et al*., 1994). Consequently, these polysaccharides, although distinguishable as receptors, react similarly as antigens.

The recent identification of the gene cluster for type 2Gn RPS of *S. gordonii* 38 (Xu *et al*., 2003) has provided insight into the molecular basis of RPS structure. The first four genes in this cluster are homologues of the common regulatory genes found in the capsular polysaccharide (CPS) gene clusters of *Streptococcus pneumoniae* (Jiang *et al*., 2001). The remaining 10 genes encode seven putative glycosyltransferases, the number required for synthesis of a lipid-linked heptasaccharide repeating unit, a repeat unit transporter (Wzx), a polysaccharide polymerase (Wzy) and galactofuranose mutase (Glf), the enzyme that supplies UDP-Gal*f*, one of five essential RPS precursors. The first glycosyltransferase is a homologue WchA, the enzyme of *S. pneumoniae* that initiates CPS biosynthesis by transferring Glc-1-PO_4 from UDP-Glc to carrier lipid (Kolkman *et al*., 1997). The similar transfer of Glc-1-PO_4 to carrier lipid in *S. gordonii* along with the presence of one Glc unit per heptasaccharide repeat defines the biosynthetic repeating unit of type 2Gn RPS. Synthesis of the recognition region of this repeating unit (i.e. the Gal*f*β1-6GalNAcβ1-3Galα1-PO_4^- portion) may depend on *wefC*, *wefD* and *wefE*. Thus, WefC may be a Galα1-PO_4 transferase based on its weak homology with a putative ManNAcα1-PO_4 transferase of *Neisseria meningitidis*, WefD may form the β1-3 linkage between GalNAc to Gal based on its predicted inverting mechanism of action, and WefE, a putative Gal*f* transferase, may transfer Gal*f* to β-GalNAc to complete synthesis of the type 2Gn heptasaccharide repeat (Xu *et al*., 2003).

The gene cluster of *S. oralis* J22 for biosynthesis of type 2G RPS has now been identified thereby providing a basis for comparative molecular studies to identify the genes for the different host-like recognition motifs in type 2G and 2Gn RPS. This was accomplished in the current study by the structural characterization of genetically engineered polysaccharides. Moreover, the reactions of these polysaccharides as receptors and antigens provide new insight into molecular basis of RPS function.

Results

Comparison of the type 2G RPS gene cluster of S. oralis *J22 with the polysaccharide gene clusters of other streptococci*

The RPS gene clusters of *S. gordonii* 38 and *S. oralis* J22, although closely related, are located between different genes in the streptococcal chromosome (Fig. 1). The *S. gordonii* 38 gene cluster is between *nrdG* and *orfO* while the *S. oralis* J22 cluster is between *dexB* and *aliA*, the same genes that flank the CPS gene clusters of *S. pneumoniae* (García *et al*., 2000). Insertion sequences

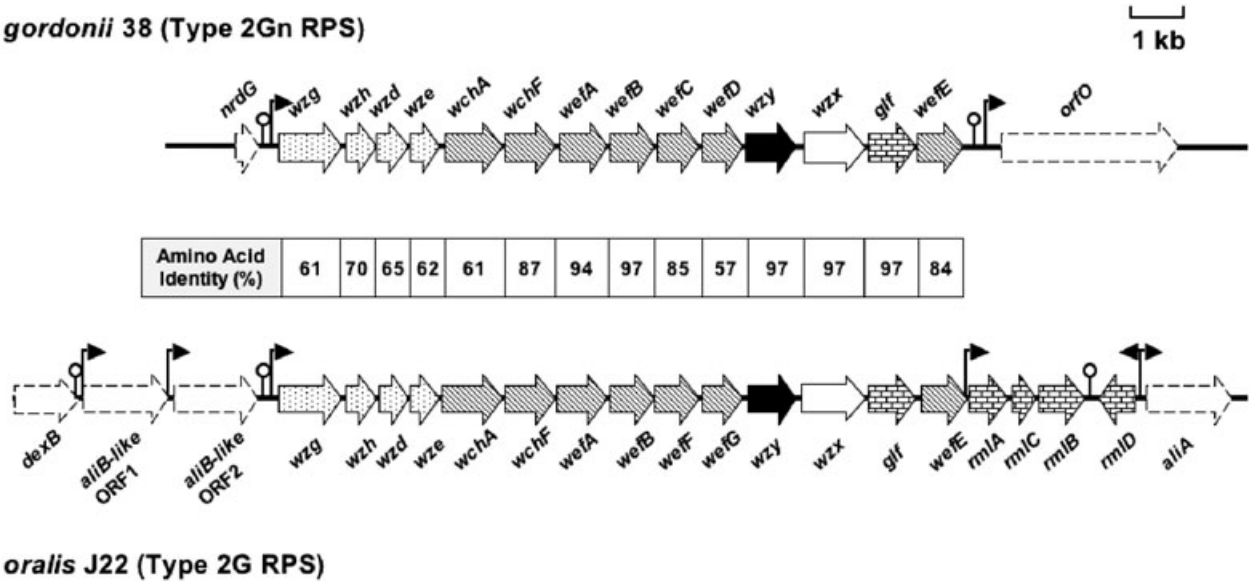

Fig. 1. ORF diagrams of the type 2Gn RPS gene cluster of *S. gordonii* 38 and type 2G RPS gene cluster of *S. oralis* J22 indicating amino acid sequence identities of proteins encoded by corresponding genes and their putative roles in RPS biosynthesis. Each cluster contains four common regulatory genes (▭), seven genes that encode putative glycosyltransferases (▨) and additional genes for a putative polysaccharide polymerase (▬), a repeat unit transporter (▭) and enzymes for nucleotide sugar biosynthesis (▭). Flanking genes (▭) are also identified as are the positions of putative promoters (▸) and rho-independent transcriptional terminators (♀).

commonly separate *dexB* and *wzg* (i.e. *cpsA*) in *S. pneumoniae*. However, these genes in *S. oralis* J22 are separated by two *aliB*-like open reading frames (ORFs), similar to those recently identified in certain non-encapsulated *S. pneumoniae* strains (Hathaway *et al*., 2004). The RPS and CPS gene clusters of these streptococci are also distinguished by the extent of their association with the four genes for dTDP-L-Rha biosynthesis. In Rha-containing serotypes of *S. pneumoniae*, these genes occur in the order *rmlA*, *rmlC*, *rmlB* and *rmlD* at the 3′-end of CPS gene clusters (Morona *et al*., 1999; Jiang *et al*., 2001). However, in *S. gordonii* 38, *rmlA*, *rmlC* and *rmlB* are transcribed from a separate operon along with *galE2*, the gene for a bifunctional epimerase that supplies both UDP-Gal and UDP-GalNAc. This operon is not associated with *rmlD*, which is transcribed independently (Xu *et al*., 2003). The arrangement of the *rml* genes in *S. oralis* J22 is transitional between that seen in *S. pneumoniae* and *S. gordonii*. Thus, *rmlA*, *rmlC* and *rmlB* are the last three genes in the *S. oralis* gene cluster and are followed by *rmlD*, which is transcribed in the opposite direction, presumably from a putative bidirectional promoter between this gene and *aliA*. Interestingly, the same arrangement of the *rml* genes and *aliA* has been noted in a strain of *S. pneumoniae* (Morona *et al*., 1999).

The first six genes in the RPS gene clusters of *S. gordonii* 38 and *S. oralis* J22 are homologues of those in the CPS gene clusters of *S. pneumoniae* serotypes 18C and 23F (Jiang *et al*., 2001). These include the four regulatory genes at the 5′-end of each cluster and the first two genes for glycosyltransferases (i.e. *wchA* and *wchF*). The homology seen over this region is greater between *S. pneumoniae* and *S. oralis* J22 (i.e. from 73% to 87% identity at the level of predicted amino acid sequence) than between *S. gordonii* 38 and *S. oralis* J22 (Fig. 1). However, homology between the RPS gene clusters of these strains jumps from 61% identity for *wchA* to 87% identity for *wchF* and remains high (i.e. from 85% to 97% identity) for seven of the eight downstream genes. The only exception is *wefD* (57% identity) which, along with *wefC*, may account for synthesis of the Gn recognition motif in type 2Gn RPS. Consequently, the corresponding genes in *S. oralis* J22 may not be *wefC* and *wefD*, but instead, different genes associated with synthesis of the G recognition motif in type 2G RPS.

Identification of the genes for synthesis the recognition motifs in type 2Gn and 2G RPS

We replaced *wefC* and *wefD* in *S. gordonii* 38 with the corresponding genes from *S. oralis* J22 to test the hypothesis that these genes direct synthesis of the distinct recognition motifs in type 2Gn and 2G RPS. Initially, *wefC* and *wefD* were replaced by the *ermAM* cassette to obtain *S. gordonii* GC15. This strain, which failed to bind RPS-specific immunoglobulin G (IgG) in dot immunoblotting (Fig. 2), was then transformed with a polymerase chain reaction (PCR) product that contained the corresponding genes from *S. oralis* J22, which are now designated *wefF* and *wefG*, flanked by targeting sequences for *S. gordonii* 38 *wefB* and *wzy*. Screening of approximately 10 000 transformants by colony immunoblotting with RPS-specific IgG resulted in the identification of one immunoreactive clone, designated *S. gordonii* GC16. DNA sequencing of this clone, in the region between *wefB* to *wzy*, established the precise replacement of *wefC* and *wefD* in the type 2Gn RPS gene cluster of *S. gordonii* 38 with *wefF* and *wefG* from *S. oralis* J22. Binding of RPS-specific IgG to *S. gordonii* GC16 was similar to that seen to wild-type *S. oralis* J22 (Fig. 2).

The ^{1}H-nuclear magnetic resonance (NMR) spectrum recorded for the RPS isolated from *S. gordonii* GC16 was identical to that of previously characterized type 2G RPS of *S. oralis* J22 (Abeygunawardana *et al*., 1990; Abeygunawardana and Bush, 1993). The structural reporter resonances in the spectra of these polysaccharides are identical in both the anomeric region (5.5–4.5 p.p.m.) and methyl region (2.2–1.2 p.p.m.). In addition, the same ^{1}H and ^{13}C signals were seen in the heteronuclear single-quantum coherence (HSQC) spectra of the RPS from *S. gordonii* GC16 (results not shown) and *S. oralis* J22 (Table S1). The RPS isolated from these two strains also reacted identically in immunodiffusion with rabbit antiserum R49 against *S. oralis* J22 (results not shown), which readily distinguished type 2G from type 2Gn RPS (Fig. 3). Finally, the presence of a G-type RPS on *S. gordonii* GC16 was indicated by differential binding of *A. naeslundii* 12104 and *S. sanguis* SK1 in co-aggregation and bacteria overlay experiments (Fig. 4). In these experiments, *A. naeslundii* 12104, which recognizes Gn- and G-containing receptors, bound type 2G RPS of *S. oralis* J22 and *S. gordonii* GC16 as well as type 2Gn RPS of *S. gordonii* 38 while *S. sanguis* SK1, which has a Gn-specific adhesin, only bound type 2Gn RPS of *S. gordonii* 38. Thus, the replacement of *wefC* and *wefD* in *S. gordonii* 38 with *wefF* and *wefG* from *S. oralis* J22 switched RPS production from type 2Gn to 2G. This result not only identifies the genes for the different recognition motifs in these polysaccharides, but also implies functional identity between the remaining five genes for glycosyltransferases in the type 2Gn and 2G RPS gene clusters of *S. gordonii* 38 and *S. oralis* J22 respectively (Fig. 1).

Contributions of individual genes to RPS structure and function

Replacement of *wefC* in *S. gordonii* 38 or *wefF* in *S. gordonii* GC16 with *ermAM* abolished cell surface RPS

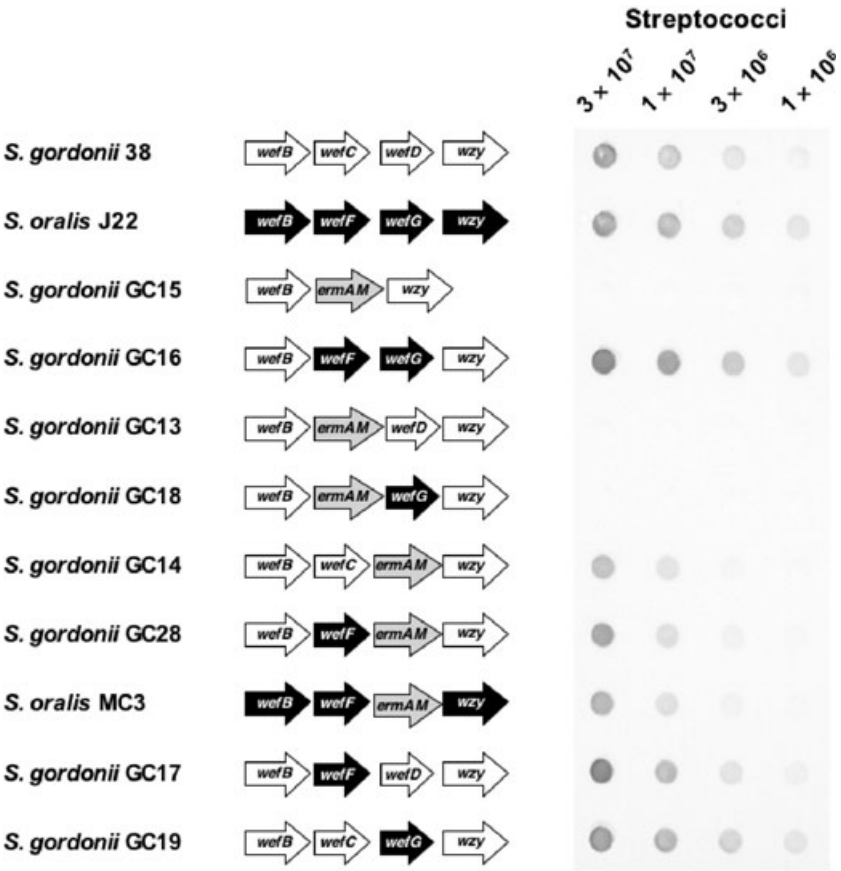

Fig. 2. Dot immunoblotting showing the reaction of RPS-specific IgG with decreasing numbers of wild-type and mutant streptococci spotted on nitrocellulose. This antibody did not react with *S. gordonii* GC15, GC13 or GC18. The partial ORF diagram of the RPS gene cluster in each strain indicates the presence of genes from *S. gordonii* 38 (⇨), *S. oralis* J22 (➡) or *ermAM* (⟹).

production as shown by failure of RPS-specific IgG to bind either resulting mutant (i.e. *S. gordonii* GC13 or *S. gordonii* GC18, respectively, in Fig. 2). The expression of downstream genes in these mutants (i.e. *wefD* in *S. gordonii* GC13 and *wefG* in *S. gordonii* GC18) was detected by reverse transcription polymerase chain reaction (RT-PCR) (results not shown), thereby suggesting that the loss of cell surface RPS was not due to polar effects of the *ermAM* insertions.

Surprisingly, *ermAM* replacement of *wefD* or *wefG* did not abolish binding of RPS-specific IgG to the resulting mutants (*S. gordonii* GC14 or *S. gordonii* GC28, respectively, in Fig. 2). The end points of these reactions were, however, from three- to ninefold lower than those of the corresponding parental strains (i.e. *S. gordonii* 38 and *S. gordonii* GC16, respectively, in Fig. 2). The yields of soluble RPS isolated from mutanolysin digests of mutant cell walls were also low. A small sample of polysaccharide was isolated from each mutant; however, the purity of the sample from *S. gordonii* GC28 was not suitable for structural analysis. To circumvent this problem, an equivalent mutant was prepared by *ermAM* replacement of *wefG* in *S. oralis* J22, a parental strain that typically yields large amounts of RPS (Cisar *et al.*, 1997). The resulting mutant (*S. oralis* MC3) and *S. gordonii* GC28 gave comparable reactions in RPS-specific dot immunoblotting (Fig. 2); however, the yield of mutant polysaccharide was greater from the former strain, presumably because cell walls of this strain are more susceptible to mutanolysin digestion. Immunodiffusion experiments performed with the purified mutant polysaccharide of *S. oralis* MC3 revealed a single antigen, identical to one of two components present in the polysaccharide preparation of *S. gordonii* GC28 (results not shown).

The presence of a novel hexasaccharide repeating subunit in the mutant polysaccharide of *S. oralis* MC3 was initially suggested by the appearance of six resonances in the anomeric region of the ^{1}H-NMR spectrum and confirmed by the appearance of six C–H resonances in the corresponding region of the HSQC spectrum (Fig. 5). The signals in the spin systems of each of the six sugar residues were identified by ^{1}H–^{1}H correlation using homonuclear coherence spectroscopy (COSY), homonuclear total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) with confirmation by heteronuclear multiple bond correlation (HMBC) and HSQC-TOCSY (data not shown) following standard methods (Abeygunawardana and Bush, 1993). The complete assignment of all ^{1}H and ^{13}C resonances are given in Table S1. Positions of glycosidic linkages were identified

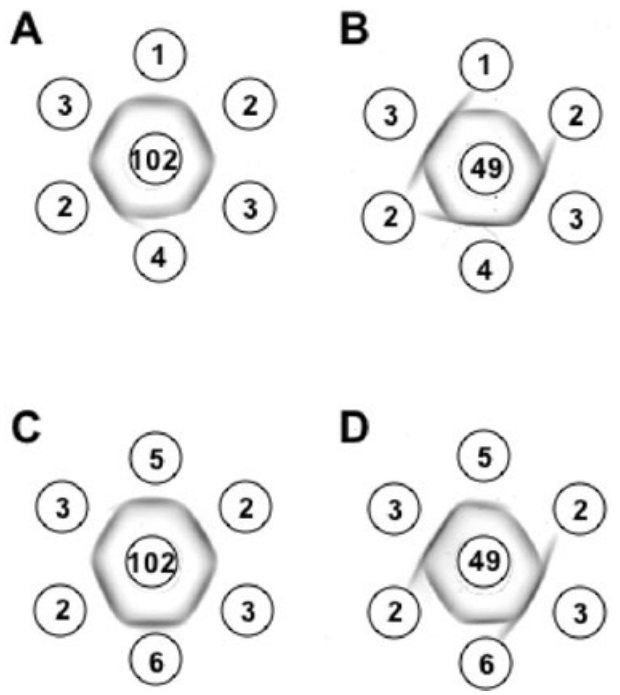

Fig. 3. Comparison of purified mutant and wild-type RPS by immunodiffusion. Centre wells were filled with antiserum R102 against *S. gordonii* 38 or antiserum R49 against *S. oralis* J22. Outer wells were filled with 0.5 mg ml^{-1} solutions of purified mutant or wild-type RPS: (1) mutant polysaccharide of *S. gordonii* GC14 (i.e. type 2Gn RPS devoid of β-GalNAc); (2) type 2Gn RPS; (3) type 2G RPS; (4) mutant polysaccharide of *S. oralis* MC3 (i.e. type 2G RPS devoid of β-Gal); (5) mutant polysaccharide of *S. gordonii* GC17 containing GalNAcβ1-3GalNAc; (6) mutant polysaccharide of *S. gordonii* GC19 containing Galβ1-3Gal.

by HMBC and confirmed by NOESY as indicated in Table 1. The similarity in chemical shifts of the *S. oralis* MC3 polysaccharide with those previously assigned for the polysaccharide of *S. oralis* J22 greatly facilitated the assignment as many could be proposed simply by chemical shift comparison then confirmed by correlation. There are some differences seen in the galactofuranoside residue, D, with larger differences in the α-GalNAc residue F. The shifts in the latter residue differ most as α-GalNAc is substituted in the 3-position in *S. oralis* J22 and in the 6-position in *S. oralis* MC3 as is shown by HMBC, NOESY and the downfield ^{13}C shift of C6. These findings indicate the following structure for the mutant polysaccharide of *S. oralis* MC3:

D F A B C
[-6Gal*f*β1-6GalNAcα1-PO_4^--6GalNAcα1-3Rhaβ1-4Glcβ1-]$_n$
Rhaα1-2┘
G

The presence of this polysaccharide on *S. gordonii* GC28 was supported by NMR analysis of the partially purified preparation obtained from this strain.

The relatively small quantity of purified polysaccharide isolated from *S. gordonii* GC14 rendered impractical the measurement of long-range ^1H–^{13}C correlation spectra such as HMBC and HSQC-TOCSY. However, the overall similarity of this polysaccharide and the *S. oralis* MC3 polysaccharide simplified the structure determination. The two polysaccharides differ only in the presence of an amide function at C2 of residue F which is α-GalNAc in *S. oralis* MC3 and α-Gal in *S. gordonii* GC14. Thus, comparison of the HSQC spectra of these polysaccharides

Table 1. Inter-residual connectivities in mutant polysaccharides produced by different streptococcal strains.

		Residue								
Strain	NMR experiment	(A) GalNAcα1→	(B) Rhaβ1→	(C) Glcβ1→	(D) Gal*f*β1→	(E) GalNAcβ1→	Galβ1→	(F) GalNAcα1→	Galα1→	(G) Rhaα1→
MC3	NOESY (300 ms)	H3(B)	H4(C)	H6(D) H6′(D)	H6(F) H6′(F)	NP[a]	NP	ND[b]	NP	H2(B)
	HMBC	C3(B)	C4(C)	C6(D) H6(D)	H6(F) H6′(F)	NP	NP	ND	NP	H2(B)
GC14	NOESY (300 ms)	H3(B)	H4(C)	H6(D) H6′(D)	H6(F) H6′(F)	NP	NP	NP	ND	H2(B)
GC17	NOESY (100 ms)	H3(B)	H4(C)	H6(D) H6′(D)	H6(E)	H3(F)	NP	ND	NP	H2(B)
	HMBC	C3(B) H3(B)	C4(C) H4(C)	C6(D) H6(D)	H6(E) H6′(E)	C3(F) H3(F)	NP	ND	NP	H2(B)
GC19	NOESY (100 ms)	H3(B)	H4(C)	H6(D) H6′(D)	ND	NP	ND	NP	ND	H2(B)
	HMBC	C3(B) H3(B)	C4(C)	C6(D) H6(D) H6′(D)	C6(E) H6(E)	NP	C3(F)	NP	ND	C2(B) H2(B)

a. Residue not present.
b. Linkage not detected by NOESY or HMBC. The phosphodiester bond between C1 of residue F and C6 of residue A was confirmed by ^1H-detected ^{31}P spin echo difference spectroscopy.

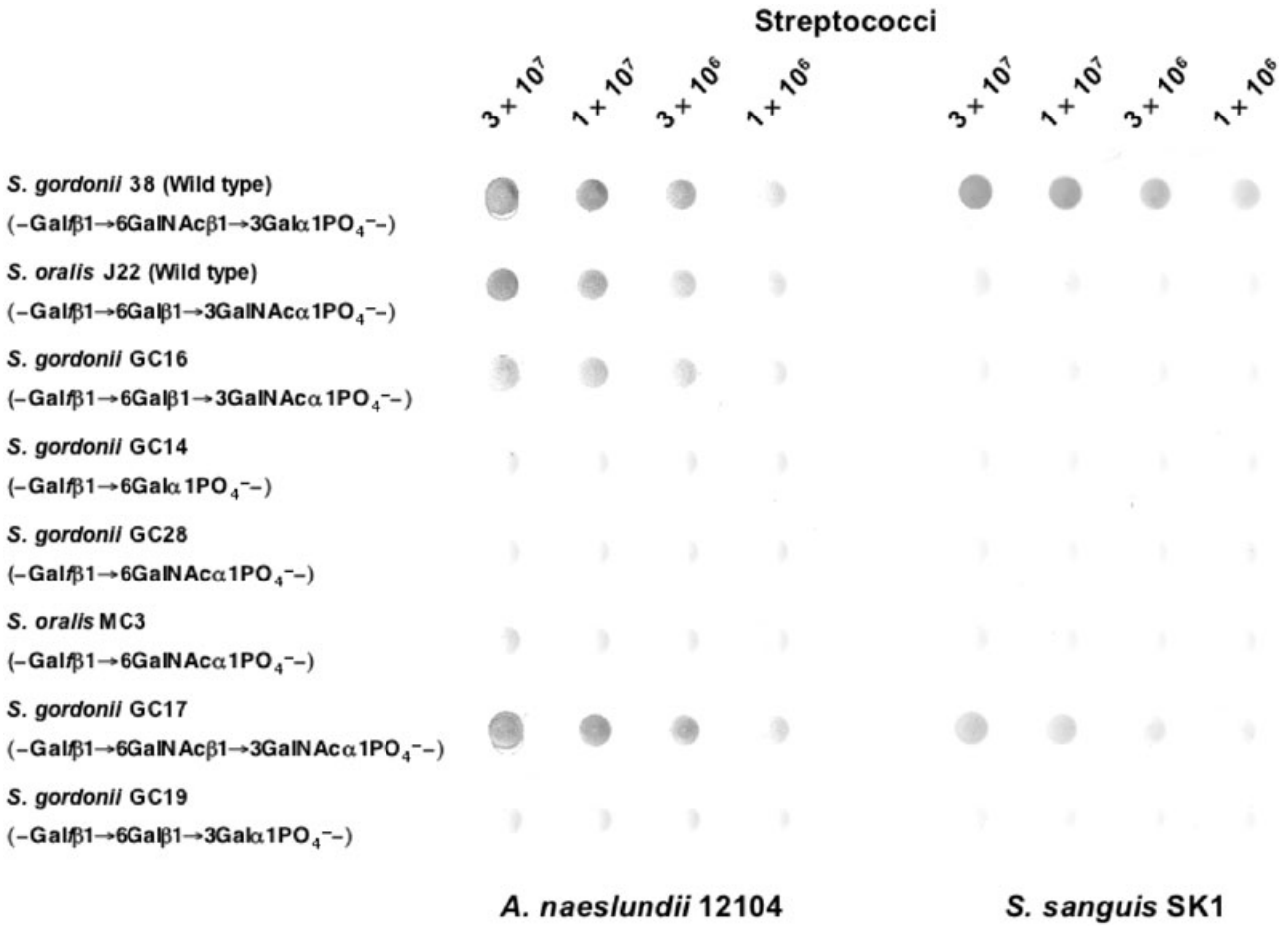

Fig. 4. Bacterial overlay experiments showing comparable binding of *A. naeslundii* 12104 or *S. sanguis* SK1 to decreasing numbers of wild-type and mutant streptococci spotted on nitrocellulose membranes. Membranes were spotted with streptococci, incubated with biotin-labelled *A. naeslundii* 12104 or *S. sanguis* SK1, washed to remove unbound bacteria and developed with avidin-HRP conjugate followed by substrate to reveal bound bacteria. The structure of the recognition region in the wild-type or mutant RPS of each immobilized streptococcal strain is indicated in parentheses. Bacterial binding was not observed to *S. gordonii* strains GC14, GC28 or GC19 or to *S. oralis* MC3.

(Fig. 5) immediately suggested assignments for most of the signals (Table S1). A few small differences do, however, exist in the chemical shifts for C–H pairs in residue D with more significant differences between the Gal or GalNAc residue F. The assignments for the *S. gordonii* GC14 polysaccharide given in Table S1 were all confirmed by ^{1}H correlation and NOESY spectra and the glycosidic linkages were confirmed by comparison of cross-peaks in the NOESY spectra of the polysaccharides of *S. gordonii* GC14 and *S. oralis* MC3. These findings indicate the following structure for the mutant polysaccharide of *S. gordonii* GC14:

D F A B C
[-6Gal*f*β1-6Galα1-PO$_4^-$-6GalNAcα1-3Rhaβ1-4Glcβ1-]$_n$
Rhaα1-2┘

Immunodiffusion performed with rabbit antisera against *S. gordonii* 38 (Fig. 3A) or *S. oralis* J22 (Fig. 3B) revealed reactions of identity between the mutant polysaccharide of *S. gordonii* GC14 lacking β-GalNAc (well 1) and type 2Gn RPS (well 2) and also between type 2G RPS (well 3) and the mutant polysaccharide of *S. oralis* MC3 lacking β-Gal (well 4). In contrast, results of the bacteria overlay technique (Fig. 4) revealed adhesin-mediated binding of *A. naeslundii* 12104 or *S. sanguis* SK1 to *S. gordonii* 38 bearing type 2Gn RPS or *S. oralis* J22 and *S. gordonii* GC16 bearing type 2G RPS but not to mutants *S. gordonii* GC14, *S. gordonii* GC28 or *S. oralis* MC3, even when as many as 9×10^7 cells of each strain were spotted on membranes (results not shown). Thus, adhesin-mediated recognition depends on the presence of immunorecessive β-GalNAc in type 2Gn RPS and immunorecessive β-Gal in type 2G RPS.

Carbohydrate engineering for the recognition motifs in type 2G and 2Gn RPS

From the association of *wefD* and *wefC* with synthesis of

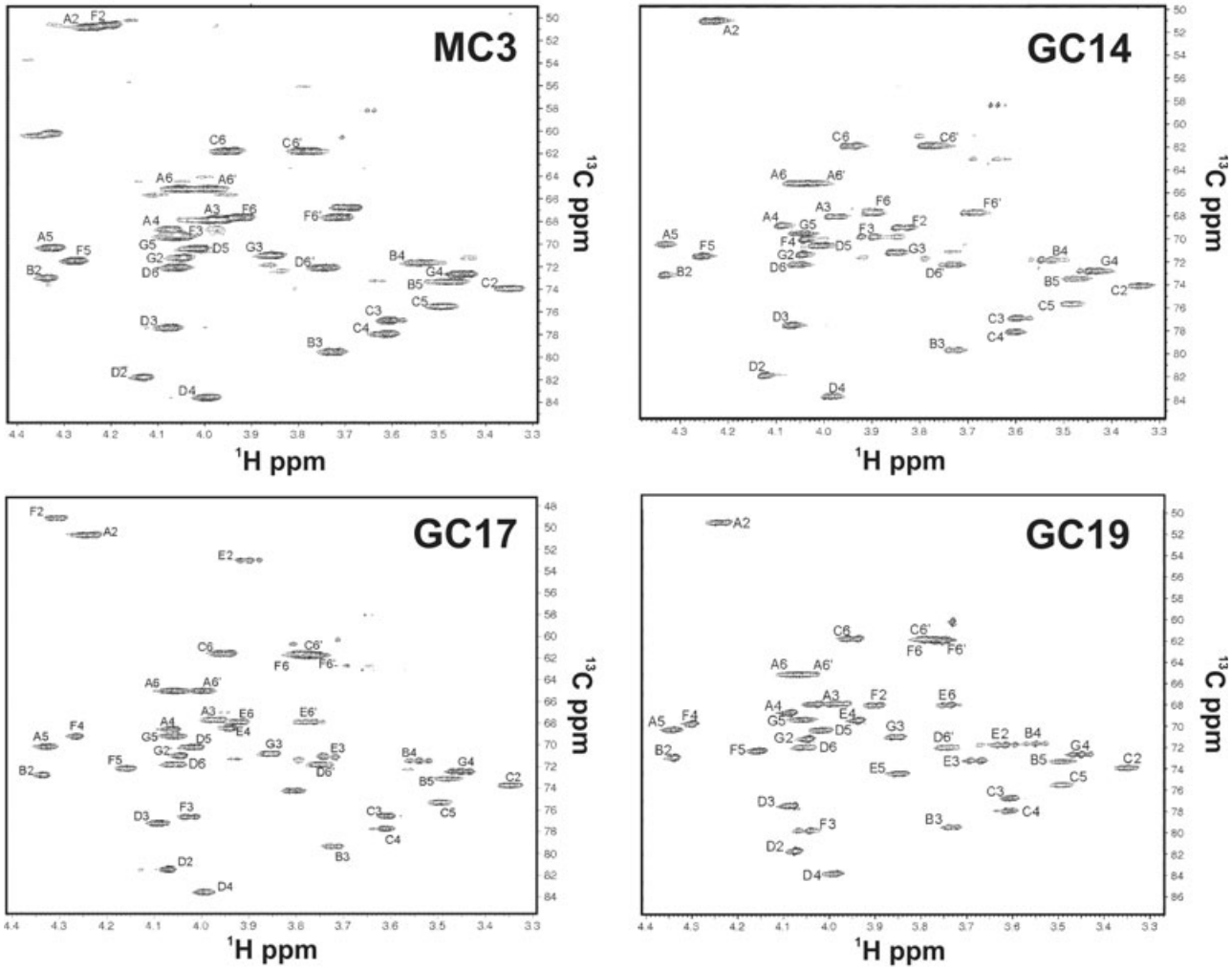

Fig. 5. Central regions of the ^{1}H–^{13}C correlation spectra (HSQC) of mutant polysaccharides from *S. oralis* MC3, *S. gordonii* GC14, *S. gordonii* GC17 and *S. gordonii* GC19. The anomeric and methyl regions are not included.

GalNAcβ1-3Galα1-PO_4^- in type 2Gn RPS and *wefG* and *wefF* with synthesis of Galβ1-3GalNAcα1-PO_4^- in type 2G RPS, we wondered whether mutant polysaccharides with GalNAcβ1-3GalNAc or Galβ1-3Gal recognition motifs could be engineered by constructing RPS gene clusters that contained *wefF* and *wefD* or *wefC* and *wefG* respectively. To examine this possibility, we transformed *S. gordonii* GC13 lacking *wefC* with an appropriately designed PCR product containing *wefF* and *S. gordonii* GC18 lacking *wefF* with an appropriately designed PCR product containing *wefC*. RPS-specific immunofluorescence of each transformed cell population revealed rare fluorescently labelled bacteria, which were isolated by RPS-specific colony immunoblotting. The results of dot immunoblotting suggested comparable production of immunoreactive cell surface polysaccharide by these mutants and the corresponding parental strains (Fig. 2, *S. gordonii* GC17 and *S. gordonii* GC19 versus *S. gordonii* 38 and *S. gordonii* GC16 respectively). DNA sequencing of the relevant region in each mutant strain confirmed the expected in-frame insertion of *wefF* in *S. gordonii* GC17 and *wefC* in *S. gordonii* GC19.

The one-dimensional ^{1}H-NMR spectra of the mutant polysaccharides isolated from *S. gordonii* strains GC17 and GC19 indicate that each polysaccharide is composed of a novel heptasaccharide repeating subunit. The HSQC spectrum of the GC17 polysaccharide (Fig. 5) shows seven signals in the anomeric C–H region and three C–H signals in the 49–55 p.p.m. region characteristic of amino sugars, presumably residues A, E and F, while the HSQC spectrum of the GC19 polysaccharide shows seven anomeric signals but only a single amino sugar, presumably residue A (Fig. 5). The assignment of C–H resonances was facilitated by the strong homology between these polysaccharides and the corresponding parental types of RPS

(Table S1). The quantities of RPS isolated from these strains were sufficient for 10 mg samples in NMR experiments allowing confirmation of all assignments by both homonuclear correlation (COSY, TOCSY, NOESY) and long-range heteronuclear correlation (HMBC, HSQC-TOCSY). In addition, all glycosidic linkages were confirmed by HMBC and NOESY spectra as documented in Table 1. The results indicate the following structures for the mutant polysaccharides of *S. gordonii* GC17 and *S. gordonii* GC19:

GC17 RPS:

D E F A B C

[-6Gal*f*β1-6GalNAcβ1-3GalNAcα1-PO_4^--6GalNAcα1-3Rhaβ1-4Glcβ1-]$_n$

Rhaα1-2┘

G

GC19 RPS:

D E F A B C

[-6Gal*f*β1-6Galβ1-3Galα1-PO_4^--6GalNAcα1-3Rhaβ1-4Glcβ1-]$_n$

Rhaα1-2┘

G

Immunodiffusion performed with a rabbit antiserum against *S. gordonii* 38 (Fig. 3C) resulted in a reaction of antigenic identity between type 2Gn RPS (well 2) and type 2G RPS (well 3). In contrast, an obvious antigenic difference between these polysaccharides was evident when the same experiment was performed with a rabbit antiserum against *S. oralis* J22 (Fig. 3D). In this experiment, a comparable antigenic difference was seen between type 2G RPS (well 3) and the mutant polysaccharide of *S. gordonii* GC19 (well 6) but not between type 2G RPS (well 3) and the mutant polysaccharide of *S. gordonii* GC17 (well 5). Thus, the antigenic difference detected between type 2G and 2Gn RPS appears to involve the presence of GalNAcα1-PO_4^- in the former polysaccharide versus Galα1-PO_4^- in the latter.

The lectin-like interaction of *A. naeslundii* 12104 with type 2Gn RPS of *S. gordonii* 38 or type 2G RPS of *S. oralis* J22 was readily demonstrated by visual assays for co-aggregation and also by the binding of biotinylated *A. naeslundii* 12104 to streptococci immobilized on nitrocellulose (Fig. 4). Interestingly, *A. naeslundii* 12104 bound to *S. gordonii* GC17 but failed to bind *S. gordonii* GC19 thereby suggesting recognition of GalNAcβ1-3GalNAc on the former strain but not Galβ1-3Gal of the latter. In similar experiments, binding of *S. sanguis* SK1, which depends on a GalNAc-specific adhesin, was more pronounced to *S. gordonii* 38 than to *S. gordonii* GC17 suggesting a preference for GalNAcβ1-3Gal in type 2Gn RPS over GalNAcβ1-3GalNAc in the mutant polysaccharide of the latter strain.

Discussion

Significant insights into the molecular basis of RPS structure and function were gained in the present investigation from the production and characterization of different genetically modified polysaccharides. These polysaccharides were engineered by replacing specific genes in the type 2Gn RPS gene cluster in *S. gordonii* 38 with the *ermAM* cassette followed by replacement of this cassette with complementary genes from the type 2G RPS gene cluster of *S. oralis* J22. The first step abolished synthesis of cell surface RPS and the second step resulted in the production of a genetically modified polysaccharide that was detected by its reaction with an RPS-specific polyclonal antibody and characterized by high-resolution NMR. The NMR method used in the present study proved especially useful for this purpose. The data of Table S1 show that modest differences in structure lead to localized differences in the C–H resonances. Thus, inspection of an HSQC spectrum readily suggested a tentative spectral assignment. For novel structures, the expected correlations were verified by homo- and heteronuclear correlation experiments and the intersaccharide linkages determined by long-range correlation. The combined results of molecular and structural studies show that deletion of *wefD* eliminated β-GalNAc from type 2Gn RPS, that deletion of *wefG* eliminated β-Gal from type 2G RPS and that swapping *wefC* and *wefF* yielded mutant polysaccharides with GalNAcβ1-3GalNAcα1-PO_4^- or Galβ1-3Galα1-PO_4^- recognition motifs respectively. These findings clearly identify the genes for the recognition motifs in types 2Gn and 2G RPS and also provide insight into the acceptor specificities of the encoded glycosyltransferases.

The previously summarized results provide clear evidence that both GalNAcα1-PO_4^- and Galα1-PO_4^- are acceptors for the WefD-mediated transfer of β-GalNAc and WefG-mediated transfer of β-Gal. The relaxed specificity of WefD and WefG for these acceptors does not, however, exclude the possibility that each glycosyltrasferase has a preference for one acceptor structure over the other. Clearly, further enzymatic characterization of these proteins is needed to assess this possibility. In contrast, with the relaxed acceptor specificity of WefD and WefG, the strict donor specificity of these and other glycosyltransferases involved in RPS biosynthesis is evident from the NMR spectra of different wild-type or mutant polysaccharides, which indicate structural homogeneity of the oligosaccharide repeating subunits in these molecules. Strict donor specificity of the GalNAc transferases involved in type 2Gn RPS biosynthesis (i.e. WefA and WefD) is also evident from the absence of detectable cell surface RPS on a previously described mutant of *S. gordonii* 38, which synthesizes all necessary RPS precursors except UDP-GalNAc (Xu *et al*., 2003).

The production of type 2G RPS following the replacement of *wefC* and *wefD* in *S. gordonii* 38 with *wefF* and *wefG* from *S. oralis* J22 associates the putative Gal*f* transferase encoded by *wefE* with the synthesis of both Gal*f*β1-6GalNAcβ in type 2Gn RPS (Xu *et al*., 2003) and Gal*f*β1-6Galβ in type 2G RPS. Moreover, WefE transfers Gal*f* to either Galα1-PO_4^- in *S. gordonii* GC14, which lacks *wefD*, or GalNAcα1-PO_4^- in *S. oralis* MC3, which lacks *wefG*, resulting in mutant polysaccharides with hexasaccharide repeating subunits. The presence of such repeats in type 2Gn or 2G RPS is not evident from the NMR spectra of these wild-type polysaccharides. Thus, the efficient WefD-dependent transfer of β-GalNAc to Galα1-PO_4^- or WefG-dependent transfer of β-Gal to GalNAcα1-PO_4^- in wild-type strains appears to prevent the WefE-dependent transfer of Gal*f* to these acceptors.

The presence of hexasaccharide repeats in the mutant polysaccharides of *S. gordonii* GC14 and *S. oralis* MC3 clearly indicates that the action of Wzy is not limited to wild-type heptasaccharide repeating subunits. Similarly, Wzy encoded by a gene in the extracellular polysaccharide (EPS) gene cluster of *Streptococcus thermophilus* Sfi6 was previously found to catalyse polymerization of a branched tetrasaccharide synthesized in *S. thermophilus* as well as a linear trisaccharide synthesized in *Lactococcus lactis* (Stingele *et al*., 1999). The yield of wild-type EPS from *S. thermophilus* was, however, significantly greater than that of mutant EPS from *L. lactis*. Likewise, the cell surface production of wild-type RPS by *S. gordonii* 38 or *S. oralis* J22 was significantly greater than the production of mutant polysaccharide by *S. gordonii* GC14 or *S. oralis* MC3 respectively. Further studies are needed to determine whether the production of these mutant polysaccharides is limited by the inefficient synthesis of lipid-linked hexasaccharide subunits or by the inefficient flipping or polymerization of these subunits from the action of Wzx or Wzy respectively.

Molecular mimicry of host glycoconjugates by the surface carbohydrates of mucosal pathogens may contribute to evasion of the host response as well as to the production of anti-host antibody (Moran *et al*., 1996). The production of such antibodies in response to normal oral colonization of RPS-bearing streptococci has not been detected (J.O. Cisar, unpublished). We have, however, noted that the reactions of certain rabbit anti-streptococcal sera, most notably R49, distinguish type 2G from 2Gn RPS (Cisar *et al*., 1997), thereby indicating detection of an epitope(s) that is closely associated with the host-like features of these polysaccharides. The present findings (Fig. 3) indicate that this epitope depends on the presence of GalNAcα1-PO_4^- in type 2G RPS but not β-Gal. Thus, the antigenic region of this polysaccharide appears to extend to the edge of the host-like recognition motif.

The specificity of *A. naeslundii* type 2 fimbriae-mediated adhesion has been assessed by the binding of bacteria to immobilized glycolipids (Brennan *et al*., 1987; Strömberg and Karlsson, 1990) and neoglycoproteins (Ruhl *et al*., 1996) and also by saccharide inhibition of the co-aggregations observed between strains of *A. naeslundii* and RPS-bearing streptococci (McIntire *et al*., 1982; 1988; Cisar *et al*., 1997). The results of these studies suggest that recognition of GalNAcβ1-3Gal and Galβ1-3GalNAc depends on the common features of these isomeric structures. This interpretation is consistent with the type 2 fimbriae-mediated recognition of GalNAcβ1-3GalNAc, demonstrated previously by the binding of *A. naeslundii* 12104 to a glycolipid from human erythrocytes (Strömberg and Karlsson, 1990) and presently by the binding of this strain to the mutant RPS of *S. gordonii* GC17 (Fig. 4). However, *A. naeslundii* did not adhere to the mutant RPS present on *S. gordonii* GC19, which has Galβ1-3Gal motifs, thereby clearly suggesting that recognition depends on the presence of N-acetyl groups. The possibility that the N-acetyl groups in GalNAcβ1-3Gal- and Galβ1-3GalNAc are accommodated at different positions in the binding site of the *A. naeslundii* adhesin provides a simple explanation for strong binding of *A. naeslundii* to these disaccharides and GalNAcβ1-3GalNAc and the failure of this organism to bind Galβ1-3Gal. Importantly, the current findings indicate that recognition of type 2Gn and 2G RPS involves both saccharide units in the host-like motifs of these polysaccharides.

The genetic engineering of bacterial polysaccharide gene clusters has been suggested as a possible approach for altering the rheological properties of EPS produced by lactic acid bacteria (Kleerebezem *et al*., 1999; Jolly *et al*., 2002; Welman and Maddox, 2003). The application of this approach has now been realized for the first time to our knowledge by altering the recognition motifs in the RPS of oral viridans group streptococci. The ability to genetically engineer bacterial surface carbohydrates has a wide range of potential applications as illustrated by the prevention of toxin-based enteric disease with a recombinant strain of *Escherichia coli* engineered to surface express a toxin receptor mimic (Paton *et al*., 2000). The limits of this emerging technology will ultimately be determined by the donor and acceptor specificities of the glycosyltransferases and polymerases encoded by available genes. The structural complexity of the surface polysaccharides present on oral viridans group streptococci suggest that these bacteria represent a rich source of such genes. Moreover, the likelihood that these genes can be used to engineer novel carbohydrate structures is increased by the present finding that the acceptor specificity of certain glycosyltransferases involved in RPS biosynthesis appears to be less strict than anticipated. Further studies of streptococcal RPS gene clusters are underway both to

Table 2. Bacterial strains used in this study.

Strain	Genotype or relevant characteristic	Reference
S. gordonii 38	Wild-type strain (type 2Gn RPS)	Reddy *et al*. (1994)
S. gordonii GC13	Em^r, *S. gordonii* 38 containing *ermAM* in place of *wefC*	This study
S. gordonii GC14	Em^r, *S. gordonii* 38 containing *ermAM* in place of *wefD*	This study
S. gordonii GC15	Em^r, *S. gordonii* 38 containing *ermAM* in place of *wefC* and *wefD*	This study
S. gordonii GC16	Em^S, *S. gordonii* GC15 containing *wefF* and *wefG* in place of *ermAM*	This study
S. gordonii GC17	Em^S, *S. gordonii* GC13 containing *wefF* in place of *ermAM*	This study
S. gordonii GC18	Em^r, *S. gordonii* GC16 containing *ermAM* in place of *wefF*	This study
S. gordonii GC19	Em^S, *S. gordonii* GC18 containing *wefC* in place of *ermAM*	This study
S. gordonii GC28	Em^r, *S. gordonii* GC16 containing *ermAM* in place of *wefG*	This study
S. oralis J22	Wild-type strain (type 2G RPS)	Abeygunawardana *et al*. (1990)
S. oralis MC3	Em^r, *S. oralis* J22 containing *ermAM* in place of *wefG*	This study
S. sanguis SK1	GalNAc-binding strain	Takahashi *et al*. (2002)
A. naeslundii 12104	GalNAc- and Gal-binding genospecies 1 strain	Cisar *et al*. (1995)

identify genetic markers for oral biofilm development and to explore the limits of carbohydrate engineering in this experimental system.

Experimental procedures

Bacterial strains and culture conditions

Table 2 lists the wild-type and mutant streptococci and actinomyces that were used in this study. The streptococcus previously identified as *S. mitis* J22 is now designated *S. oralis* J22 based on results from the recent sequencing of housekeeping genes in this strain (M. Kilian, pers. comm.). The bacteria listed were routinely grown at 37°C in Todd–Hewitt broth (THB; Difco Laboratories). Erythromycin was added to a final concentration of 10 μg ml^{-1} for the cultivation of *ermAM*-containing strains. Chemically competent *E. coli* DH5α from Invitrogen was grown aerobically at 37°C in LB broth or agar, which was supplemented with 100 μg ml^{-1} ampicillin, 20 μg ml^{-1} chloramphenicol, 100 μg ml^{-1} kanamycin or 200 μg ml^{-1} erythromycin as required for the maintenance of plasmids.

Immunological methods

Rabbit antisera R102 against *S. gordonii* 38 and R49 against *S. oralis* J22 have been described (Cisar *et al*., 1997). The RPS-specific IgG used in this study was affinity-purified from antiserum R49 by 4 M $MgCl_2$ elution from a small column of immunoadsorbent containing coupled type 2G RPS as previously described (Xu *et al*., 2003).

Dot immunoblotting was performed to detect the reaction of RPS-specific IgG with streptococci spotted on nitrocellulose membranes. Streptococci were harvested from stationary-phase cultures, washed and suspended to a cell density of approximately 2×10^9 ml^{-1} in 0.02 M Tris-buffered saline (TBS) (pH 7.5) based on measurements of turbidity made with a Klett-Summerson colorimeter. Nitrocellulose membranes were spotted with decreasing numbers of streptococci using a Bio-Dot Microfiltration Apparatus (Bio-Rad) and blocked by incubation in TBS containing 0.1% Tween-20 and 2% skim milk. Membranes were then incubated with RPS-specific IgG (50 ng ml^{-1}) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad) before development with a Metal Enhanced DAB Substrate Kit (Pierce Biotechnology) to detect bound anti-RPS antibody.

Immunodiffusion experiments were performed in 1% agarose gel cast on Gel Bond Film (FMC BioProducts). Wells were filled with undiluted rabbit antisera or 0.5 mg ml^{-1} purified RPS and incubated overnight at 4°C to allow immunoprecipitation. Gels were soaked 2 days in cold 0.5 M NaCl to remove soluble protein followed by water, dried and then stained with Coomassie blue as previously described (Cisar *et al*., 1997).

Bacterial adhesion assays

Co-aggregations between *A. naeslundii* 12104 or *S. sanguis* SK1 and RPS-bearing streptococci were assessed visually (Cisar *et al*., 1979; Hsu *et al*., 1994). A previously described bacterial overlay assay (Ruhl *et al*., 1996; 2004) was performed to compare the binding of biotin-labelled *A. naeslundii* 12104 or *S. sanguis* SK1 to different immobilized wild-type and mutant streptococcal strains. Decreasing numbers of streptococci were spotted on nitrocellulose membranes using a Bio-Dot Microfiltration Apparatus as described above. Membranes were then blocked by overnight incubation at 4°C in TBS containing 5% bovine serum albumin, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ (blocking buffer). *A. naeslundii* 12104 and *S. sanguis* SK1 were harvested from overnight cultures and labelled by incubation with 100 μg ml^{-1} sulphosuccinimidyl 6-(biotinamido) hexanoate-biotin (Pierce) for 1 h at room temperature. Pre-blocked nitrocellulose membranes were incubated with biotin-labelled bacteria (5×10^8 per ml in blocking buffer) for 3 h at 4°C, washed in blocking buffer to remove unbound bacteria and incubated with 0.2 U of avidin-D-HRP (Bio-Rad) per ml followed by DAB substrate as described above to detect bound bacteria.

Cloning and sequencing of the gene cluster for type 2G RPS

The 27 833 bp DNA sequence of the *S. oralis* J22 RPS gene cluster and flanking regions, which is available under GenBank Accession No. AB181235, was assembled from the sequences of overlapping PCR fragments. The template for

initial PCR reactions was *S. oralis* J22 chromosomal DNA, which was prepared using a Wizard Genomic DNA purification kit (Promega). Reaction mixtures also contained KOD Hot Start DNA polymerase (Novagen) and primer pairs available from previous characterization of the type 2Gn RPS gene cluster of *S. gordonii* 38 (Xu *et al*., 2003). The sequences of the PCR fragments obtained were extended when necessary by inverse PCR (Ochman *et al*., 1988). Briefly, genomic DNA from *S. oralis* J22 was digested with an appropriate restriction enzyme. After inactivation of this enzyme, the digested DNA was circularized by self-ligation and used as template for PCR performed with appropriate primers. PCR fragments were cloned in pBluescript (Stratagene), pCR4Blunt-TOPO (Invitrogen) or pMCL200 (Nakano *et al*., 1995) before DNA sequencing at Sequetech, Mountain View, USA. Two independent amplicons of each cloned DNA fragment were sequenced and compared to insure the fidelity of PCR amplification. Sequences were assembled and annotated using Vector NTI software (Invitrogen). Nucleotide and predicted amino acid sequence homologies with genes and proteins in the database were identified by BLAST (Altschul *et al*., 1990).

Construction of streptococcal mutant strains

The *ermAM* mutant strains listed in Table 2 were constructed by transformation of wild-type streptococci with DNA constructs containing the *ermAM* cassette flanked by targeting sequences for the streptococcal gene of interest. The three DNA sequences (i.e. upstream targeting sequence, *ermAM* cassette and downstream targeting sequence) were linked by overlap extension PCR (Horton *et al*., 1989; Lee and Morrison, 1999) performed using KOD Hot Start DNA polymerase. The primers used to amplify the *ermAM* cassette from pKSerm2 (Lunsford and London, 1996) and each targeting sequence (approximately 0.7 kb) from streptococcal genomic DNA were designed to create nucleotide sequence complementarity between the 3′-end of the upstream targeting sequence and the 5′-end of the *ermAM* cassette and also between the 3′-end of the *ermAM* cassette and the 5′-end of the downstream targeting sequence. Overlap extension PCR was then performed using the three DNA fragments as mixed template and primers designed from the 5′-end of the upstream targeting sequence and the 3′-end of the downstream targeting sequence. Transformation of *S. gordonii* 38 and related mutant strains with overlap extension PCR products was performed as previously described (Lunsford, 1995) except that the transformation medium contained 5% heat-inactivated horse serum (Sigma-Aldrich) in place of 0.15% filter sterilized bovine serum albumin. Selection of transformants was by anerobic growth on plates of brain–heart infusion agar (BHI, Difco) containing 10 μg ml^{-1} erythromycin. The location of the *ermAM* cassette in each insertional mutant strain (Table 2) was verified by the amplification of specific PCR products across the upstream and downstream boundaries of the *ermAM* insertion, using primers for chromosomal sequences that were extraneous to those present in the transforming DNA.

Streptococcus gordonii GC16, GC17 and GC19 were obtained by replacement of the *ermAM* cassette in *S. gordonii* GC15, GC13 and GC18, respectively, with intact genes of *S. oralis* J22. This was accomplished by transformation of each *ermAM*-containing strain with an overlap extension PCR product that contained the *S. oralis* J22 gene(s) of interest flanked by targeting sequences for precise in-frame replacement of the *ermAM* cassette. Transformants were identified by their reaction with RPS-specific IgG in colony immunoblotting. Briefly, transformation reactions were diluted, spread on BHI plates and incubated anaerobically for 1 day at 37°C to allow growth of isolated colonies. A sterile nitrocellulose membrane was then placed on each plate in contact with the colonies. The membranes were carefully removed so as not to disturb the underlying colonies and processed by for binding of RPS-specific IgG (50 ng ml^{-1}) to adsorbed bacteria by procedures that were essentially the same as those described for dot immunoblotting.

Isolation of RPS

Streptococcal RPS was purified as previously described (Cisar *et al*., 1997) with minor modifications. Briefly, bacteria harvested from 12–16 l of stationary-phase cultures were treated with 0.1% Triton X-100 to disrupt membranes and then digested with Ribonuclease A followed by *Streptomyces griseus* Protease (both enzymes were from Sigma-Aldrich) to facilitate the removal cytoplasmic material. Surface polysaccharides were solubilized by mutanolysin (Sigma-Aldrich) digestion of the resulting crude cell walls. Protein was precipitated from mutanolysin digests by the addition of cold trichloroacetic acid to a final concentration of 5%. The soluble fraction was neutralized and dialysed to remove salt before purification of RPS by gradient elution from a DEAE Sephacel anion exchange column. Purified polysaccharides were detected by immunodiffusion and assays for total carbohydrate in fractions that contained from 120 mM to 160 mM NaCl.

Nuclear magnetic resonance analysis

Nuclear magnetic resonance spectra were recorded on a Bruker DRX 500 MHz spectrometer with a cryoprobe using XWINNMR as the standard acquisition software. The NMR measurements were performed at 25°C. Generally, a 10 mg sample of RPS (4 mg of the GC14 sample) was exchanged twice with 3 ml of 99.96% D_2O, lyophilized and dissolved in 0.6 ml of 99.99% D_2O. Chemical shifts were recorded relative to internal acetone (^{1}H, 2.225 p.p.m.; ^{13}C, 31.05 p.p.m.). All the data were processed using NMRPIPE, NMRDRAW and NMRVIEW software. Double-quantum filtered COSY and TOCSY with a spin lock of 70 ms were carried out to assign the scalar coupled proton of the same monosaccharide residue. For assignment of the ^{13}C chemical shift, HSQC and HSQC-TOCSY with a spin lock of 70 ms were used. Interresidual linkages were determined by NOESY with mixing times of 100 ms and 300 ms. HMBC with a delay of 50 ms for evolution of long-range coupling was used to further validate the linkages and for confirmation of the assignments.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. ^{1}H and ^{13}C chemical shifts in NMR spectra of polysaccharides from different streptococcal strains.