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Complete Structure of the Cell Surface Polysaccharide of *Streptococcus oralis* C104: A 600-MHz NMR Study†

Chitrananda Abeygunawardana and C. Allen Bush*
Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228
John O. Cisar
Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, Maryland 20892
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ABSTRACT: Specific lectin–carbohydrate interactions between certain oral streptococci and actinomyces contribute to the microbial colonization of teeth. The receptor molecules of *Streptococcus oralis*, 34, ATCC 10557, and *Streptococcus mitis* J22 for the galactose and *N*-acetylgalactosamine reactive fimbrial lectins of *Actinomyces viscosus* and *Actinomyces naeslundii* are antigenically distinct polysaccharides, each formed by a different phosphodiester-linked oligosaccharide repeating unit. These streptococci all coaggregated strongly with both *A. viscosus* and *A. naeslundii* strains, whereas *S. oralis* C104 interacted preferentially with certain strains of the latter species. Receptor polysaccharide was isolated from *S. oralis* C104 cells and was shown to contain galactose, *N*-acetylgalactosamine, ribitol, and phosphate with molar ratios of 4:1:1:1. The ^{1}H NMR spectrum of the polysaccharide shows that it contains a repeating structure. The individual sugars in the repeating unit were identified by ^{1}H coupling constants observed in E-COSY and DQF-COSY spectra. NMR methods included complete resonance assignments (^{1}H and ^{13}C) by various homonuclear and heteronuclear correlation experiments that utilize scalar couplings. Sequence and linkage assignments were obtained from the heteronuclear multiple-bond correlation (HMBC) spectrum. This analysis shows that the receptor polysaccharide of *S. oralis* C104 is a ribitol teichoic acid polymer composed of a linear hexasaccharide repeating unit containing two residues each of galactopyranose and galactofuranose and a residue each of GalNAc and ribitol joined end to end by phosphodiester linkages with the following structure.

$$[\rightarrow 6)\mathrm{Gal}f(\beta 1\rightarrow 3)\mathrm{Gal}_p(\beta 1\rightarrow 6)\mathrm{Gal}f(\beta 1\rightarrow 6)\mathrm{Gal}_p\mathrm{NAc}(\beta 1\rightarrow 3)\mathrm{Gal}_p(\alpha 1\rightarrow 1)\text{ribitol}(5\rightarrow \mathrm{PO}_4^-]_n$$

The viridans streptococci that colonize teeth (Nyvad & Kilian, 1987; Kilian et al., 1989) frequently have cell surface polysaccharides that serve as receptor molecules for the galactose and *N*-acetylgalactosamine reactive lectins of various other oral bacteria including *Actinomyces viscosus* and *Actinomyces naeslundii* (Cisar, 1986). Structural studies of the

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antigenically distinct receptor polysaccharides of *Streptococcus oralis* 34[1] (McIntire et al., 1987; Abeygunawardana et al., 1989), *Streptococcus mitis* J22 (Abeygunawardana et al., 1990), and *Streptococcus oralis* ATCC 10577 (Abeygunawardana et al., 1991) have shown that each is composed of a different hexa- or heptasaccharide repeating unit linked end to end by phosphodiester bonds. Each repeating unit included Gal$_f$(β1→6)Gal(β1→3)GalNAc(α1→) or Gal$_f$(β1→6)GalNAc(β1→3)Gal(α1→), and recognition of these structures by the lectins of *Actinomyces* spp. was postulated to depend on the exposure of internal Gal(β1→3)GalNAc or GalNAc(β→3)Gal, respectively by the flexible (β1→6) linkage from Gal$_f$. Whereas streptococcal strains 34, J22, and ATCC 10557 all coaggregated strongly with many strains of both *A. viscosus* and *A. naeslundii*, *S. oralis* C104 was distinguished by its preferential interaction with certain *A. naeslundii* strains (Cisar et al., 1979). Further studies (Kolenbrander & Williams, 1983) identified a number of other streptococcal strains that participitated in lactose-sensitive coaggregations with strains of *A. naeslundii* such as ATCC 12104 (WVU45) but failed to interact with various strains of *A. viscosus* including strain T14V. A difference between these actinomyces was also inferred from the relative abilities of certain saccharides to inhibit their lectin-mediated coaggregations with *S. oralis* 34 (McIntire et al., 1983).

To provide a further insight into the specificity of lectin-mediated interbacterial adherence, the receptor polysaccharide of *S. oralis* C104 was isolated and examined by high-resolution NMR to determine its structure.

Material and Methods

Isolation and Biological Characterization. The cell wall polysaccharide of *S. oralis* C104 was isolated by methods similar to those described previously for the other strains (Abeygunawardana et al., 1990, 1991). Briefly, crude cell walls were prepared from bacteria cultured in 20 L of complex media and digested with mutanolysin (M-3765, Sigma Chemical Co., St. Louis, MO) to solubilize the polysaccharide. The soluble fraction (900 mg dry weight), obtained after precipitation of protein in the presence of cold 5% trichloroacetic acid, was applied to a column of DE52 anion exchanger (Whatman BioSystems Ltd., Maidstone, Kent) equilibrated with 10 mM sodium phosphate, pH 8.0, and eluted with this buffer followed by a gradient (0–100 mM) of KCl in starting buffer. The C104 receptor polysaccharide emerged as the most anionic component in the extract and was recovered from fractions containing 75–100 mM KCl. The polysaccharide was detected by an inhibition of hemagglutination assay using the lectin of *Bauhinia purpurea* (BPA, L-2501, E.Y. Labs, Inc., San Mateo, CA or Sigma L-6013) with human O RBC and also by immunodiffusion with rabbit antiserum against *S. oralis* C104 whole cells. The polysaccharide was applied to a column of Sephacryl S400 (Pharmacia) in 0.15 M NaCl, recovered in fractions midway between the void and total volumes, applied to a column of Q Sepharose (Pharmacia) equilibrated with 10 mM potassium maleate buffer (pH 2.5), and eluted with a gradient (0–200 mM) of KCl prepared in this buffer. A peak of inhibitory activity for BPA-hemagglutination was detected in the fractions containing 145–180 mM KCl. This material (approximately 20 mg) reacted in immunodiffusion with anti-*S. oralis* C104 serum to form a single sharp line of precipitation and gave no reaction with antisera against *S. oralis* 34, *S. mitis* J22, or *S. oralis* ATCC 10557 (results not shown). Similarly, the anti-*S. oralis* C104 serum did not precipitate the receptor polysaccharides of the latter three strains.

Sugar Composition. The carbohydrate composition of the polysaccharide was determined by HPLC as perbenzoylated methyl glycosides (monosaccharides) according to the method of Jentoft (1985) and also by direct analysis of acid hydrolysate of polymer by high-performance anion exchange chromatography (HPAE). Polysaccharide sample (0.5 mg) was methanolyzed with 1 N HCl in MeOH at 80 °C for 4 h. Following re-*N*-acetylation of any hexosamine present, constituent methyl glycosides were perbenzoylated as described earlier (Abeygunawardana et al., 1990). The peaks (UV absorbance at 230 nm) in the reverse-phase chromatogram (Spherisorb C-18 ODS II, 3 μm, 15 cm; Alltech Associates) were assigned from the retention times of perbenzoylated methyl glycosides and perbenzoylated monosaccharide alditols prepared from standard sugars.

Another sample of polysaccharide ($\approx$250 μg) was hydrolyzed with 2 N trifluoroacetic acid (200 μL, 100 °C, 4 h). After evaporation of TFA under stream of N_2, the sample was taken in to 200 μL of deionized water and analyzed by HPAE chromatographic system using a Carbopac PA1 column (4 × 250 mm) and pulsed amperometric detector (Dionex). Alditols, neutral monosaccharides, and amino sugars resulting from de-*N*-acetylation of acetamido sugars were eluted with 15 mmol NaOH at 1 mL/min. Phosphorylated monosaccharides (nonglycosidically linked phosphomonoester derivatives present in the polymer or those resulting from hydrolysis of phosphodiester linkages) were eluted with an 80:20 mixture of 100 mmol of NaOH/500 mmol of NaOAc in 100 mmol of NaOH for 10 min followed by a linear gradient of NaOAc to 40:60 for the next 20 min. Peaks were identified by comparing the retention times with that of standard monosaccharide phosphates (Sigma). Ribitol phosphate was prepared by $NaBH_4$ reduction of ribose 5-phosphate (Egan et al., 1982).

Nuclear Magnetic Resonance Spectroscopy. Spectra were recorded on a Bruker AM-600 (600.13 MHz ^{1}H) or GN-500 (500.11 MHz ^{1}H) spectrometer. The observed ^{1}H chemical shifts are reported relative to internal standard sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) with acetone as an internal standard (2.225 ppm downfield from DSS). The carbon chemical shifts are relative to internal acetone (31.07 ppm). The phosphorus chemical shifts are reported relative to the external reference signal of 85% H_3PO_4 contained in a sealed capillary tube. The polysaccharide sample (10 mg) was exchanged in D_2O (99.8 atom % *D*) and lyophilized three cycles. The NMR sample was prepared by redissolving the dried sample in 450 μL of high purity D_2O (99.96 atom % *D*).

All 2D NMR data sets were recorded at 25 °C without sample spinning. Data were acquired in the phase-sensitive mode by using the TPPI (Marion & Wüthrich, 1983) method in AM-600 or the method of States et al., (1982) in GN-500. DQF-COSY (Rance et al., 1983), E-COSY (Griesinger et al., 1985, 1987), HOHAHA (Bax & Davis, 1985), and NOESY (Kumar et al., 1980) were recorded at 600 MHz with standard pulse sequences. ^{1}H–^{13}C correlation spectra were recorded in the proton-detected mode with a Bruker 5-mm inverse broad band probe using Bruker reverse electronics. The pulse sequence used for single-bond correlation spectra (HMQC) was that of Bax et al. (1983). WALTZ-16 (Shaka et al., 1983)

[1] *Streptococcus sanguis* strains ATCC 10557, 34, C104, and J22 have been designated as *S. oralis* ATCC 10557, *S. oralis* 34, *S. oralis* C104, and *S. mitis* J22, respectively, on the basis of the taxonomic scheme of Kilian et al. (1989).

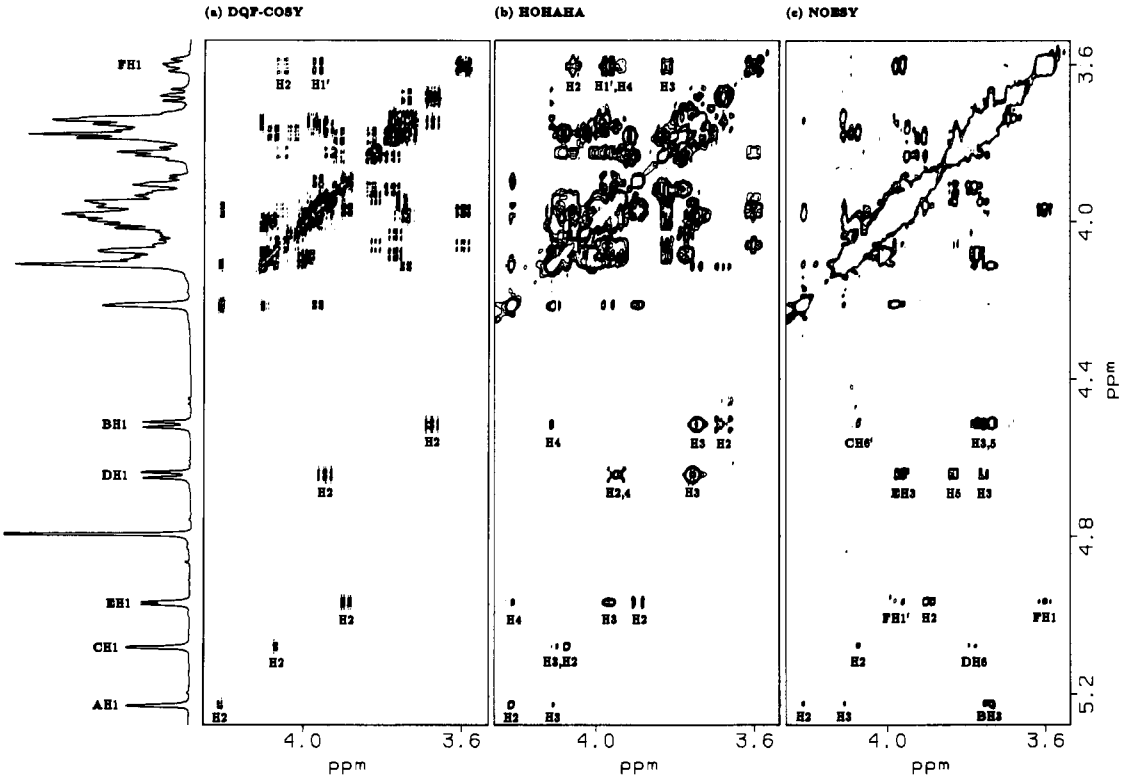

FIGURE 1: ^{1}H homonuclear two-dimensional correlation spectra of the receptor polysaccharide from *S. oralis* C104 at 600 MHz. (a) DQF-COSY spectrum, the data matrix was 512 × 4K (TPPI) complex points, with 16 scans per t_1 value. Sine bell apodizations with 10° and 20° phase shifts were used in the t_2 and t_1 dimensions, respectively. Data were zero filled in the t_1 dimension to obtain a final data matrix with 2K × 2K real points. Both positive and negative contours are shown in the plot. (b) HOHAHA spectrum (62-ms spin-lock time) and (c) NOESY spectrum (300-ms mixing time). The data matrices were 512 × 2K (TPPI) complex points with 8 and 16 scans per t_1 value for HOHAHA and NOESY, respectively. Gaussian line broadening (3 Hz) in t_2 and a cosine bell in t_1 were used prior to Fourier transformation. Spectra were zero filled in t_1 to obtain 1K × 1K real matrices. All spectra use an identical sweep width of 2202 Hz. The normal 1D spectrum is displayed at the left.

decoupling at the carbon frequency was used during the acquisition. Multiple-bond correlation spectra (HMBC) (Bax & Summers., 1986) were recorded in the phase-sensitive mode. The combination experiment HMQC-COSY (Gronenborn et al., 1989) was recorded at 500 MHz without ^{13}C decoupling during the acquisition with a 5-mm RPT probe and X-nucleus decoupling hardware in the GN-500 spectrometer. Experimental details and processing parameters of the spectra are given in the figure captions.

NMR data processing was carried out on VAX station 3200 and Silicon Graphics Personal Iris computers using the FTNMR program (Hare Research Inc. Woodinville, WA). Bruker data sets were transferred to the VAX via magnetic tape and the Bruker data transfer protocol in the FTNMR program. GN data were transferred via ethernet to these computers and converted to readable files by an inhouse program (GENET).

RESULTS

The ^{1}H NMR spectrum of *S. oralis* C104 polysaccharide in D_2O at 600 MHz (Figure 1) shows five signals (5.218, 5.071, 4.959, 4.635, and 4.507 ppm) characteristic of anomeric protons and an upfield methyl signal at 2.040 ppm indicating the presence of at least five sugar residues, one of them an acetamido sugar. The ^{13}C spectrum at 125 MHz shows five resonances at 110.05, 108.62, 103.93, 103.91, and 99.92 ppm typical of anomeric carbon resonances and signals at 53.31 and 23.21 ppm consistent with an acetamido sugar residue. The ^{13}C DEPT spectrum (Doddrell et al., 1982) recorded with 135° ^{1}H flip angle shows seven methylene carbons, indicating the presence of an alditol in addition to the aforementioned sugar residues. The ^{31}P NMR spectrum at 202 MHz shows a single phosphorus resonance at 1.45 ppm (pD 2.1). In the resolution-enhanced ^{13}C spectrum, two methylene carbon resonances (67.17 and 67.36 ppm) and a resonance at 70.03 ppm show ^{13}C–^{31}P couplings.

Carbohydrate analysis by HPAE chromatography with pulsed amperometric detection of the acid hydrolysate of the polysaccharide shows the presence of Gal, GalNH_2, and a peak that elutes early (2.70 min, 15 mM NaOH) in the chromatograms, indicating an alditol. However, we were unable to identify this residue due to the poor selectivity of retention times for several authentic monosaccharide alditols tested undeer these chromatographic conditions. The lack of selectivity and retention of mono- and oligosaccharide alditols in anion-exchange chromatography is attributed to the absence of a reducing function in these molecules (Reddy & Bush, 1991). The chromatogram recorded with 100 mmol of NaOH and linear gradient of NaOAc shows two peaks that elute late. The identities of these peaks were established as ribitol-5-PO_4 (18.1 min) and Gal-6-PO_4 (26.2 min) on the basis of retention times with that of the standard samples.

Retention times of perbenzoylated sugar derivatives in reverse-phase HPLC, on the other hand, seems to correlate with the number of *O*-benzoyl groups in the molecule. In general, perbenzoylated monosaccharide alditols have longer retention times than the corresponding benzoylated methyl glycosides. Reverse-phase chromatograms (75% acetonitrile in water) of benzoylated methyl glycosides of polysaccharide prepared according to the method of Jentoft (1985) showed 4 mol of Gal, 1 mol of GalNAc, and 1 mol of ribitol. Sugars were identified by retention times of authentic samples prepared from standard sugars and the other pentitols. Xylitol (12.0

min), arabitol and lyxitol (both at 12.2 min) have different retention times than that of ribitol (13.2 min). The ^{31}P NMR spectrum of the polysaccharide recorded at 202 MHz shows only one ^{31}P resonance (1.45 ppm, pD 2.1) characteristic of a phosphodiester linkage. These data indicated that the polysaccharide from *S. oralis* C104 is composed of a hexasaccharide (Gal/GalNAc/ribitol = 4:1:1) repeating unit polymerized through a phosphodiester linkage, which involves C-5 of ribitol and C-6 of one of the galactose residues.

In general, the relative stereochemistry and anomeric configuration of constituent monomers (hexapyranoses) in a carbohydrate polymer can be assigned by vicinal coupling constants (Altona & Hasnoot., 1980) observed in the DQF-COSY spectrum (Dabrowski et al., 1987; Berman, 1987, Abeygunawardana et al., 1990). Since all the sugar residues in the repeating unit of *S. oralis* C104 polysaccharide except ribitol have the galacto configuration, the anomeric configuration and ring size (pyranose or furanose form) of these residues can be obtained directly from coupling constants of the anomeric proton resonances. The ^1H NMR spectrum of the polysaccharide at 600 MHz shows two resonances with large coupling constants (4.635 ppm, $J_{1,2}$ 8.4 Hz; and 4.507 ppm, $J_{1,2}$ 8.0 Hz) that belong to residues having the β-galactopyranose configuration, a resonance with $J_{1,2}$ = 3.6 Hz (4.959 ppm) belonging to α-galactopyranose and two unresolved singlets at 5.218 and 5.071 ppm ($J_{1,2} \leq 2$ Hz) presumably belonging to two residues having the galactofuranose configuration. The anomeric configurations of furanose residues were assigned as β on the basis of their attached carbon resonances (discussed below).

As discussed earlier (Abeygunawardana et al., 1991), starting from the anomeric resonances, the ^1H spin systems of individual sugar residues can be assigned by homonuclear 2D spectra (Figure 1). Unlike other *Streptococcus* polysaccharides studied (Abeygunawardana et al., 1989, 1990, 1991), the ^1H NMR spectrum of the C104 polysaccharide shows most of the ring protons appearing in a narrow range (33 protons in 0.4 ppm). Tracing cross peak connectivities between vicinally coupled protons in the DQF-COSY spectrum (Figure 1a) often fails to provide complete assignment of the given spin system, due to either distorted multiplets resulting from strong coupling or partial cancellation of cross peaks due to overlap with other cross peaks or diagonal peaks. Assignments beyond strongly coupled or closely spaced resonances can be obtained from the 2D HOHAHA spectrum (Figure 1b). Cross sections taken through an isolated resonance of a particular spin system, typically an anomeric proton, can extend assignments beyond such pairs of resonances. However, both techniques fail to extend assignments through vicinal protons having small coupling constants. This can be illustrated in H4,H5 pairs ($J_{4,5} < 1$ Hz) of galactopyranose residues. Since the natural line width of proton resonances in polysaccharides is larger than this coupling constant, the H4/H5 cross peak in DQF-COSY often shows extensive cancellation resulting from the antiphase nature of the multiplet components. Due to inefficient magnetization transfer during the relatively short isotropic mixing time ($\approx$60 ms), the HOHAHA spectrum also fails to provide connectivity beyond the H4 resonance of galactopyranosides. Since the H5 and H6 protons of these residues have nonvanishing coupling constants, it is possible to find cross peaks between these protons in DQF-COSY or HOHAHA spectra. In practice, however, this can be difficult due to the fact that some of these cross peaks appear in the crowded regions of spectra and there is a lack of correlation to the rest of the spin system.

TQF-COSY (Piantini et al., 1982) spectra, which show cross peaks only for the systems having three or more mutually coupled spins, thereby simplifying crowded regions of spectra, can provide a useful method for locating the H5 and H6 protons (Homans et al., 1986; Abeygunawardana et al., 1990). The exclusive COSY (E-COSY) spectrum (Griesinger et al., 1985, 1987), which provides an attractive alternative method for separate DQF and TQF COSY spectra, can also be employed to distinguish three spin systems involving methylene protons. The results of an E-COSY experiment can be best understood as a linear combination of multiple-quantum filtered COSY spectra of different orders (p = 2, 3, etc.). It has the advantageous features of both DQF- and TQF-COSY spectra such that, for two mutually coupled spins, the cross peak appears like one in a DQF-COSY spectrum whereas, for three or more mutually coupled spins, the cross peak shows a highly simplified pattern (Figure 2b) with components resulting only from connected transitions (Griesinger et al., 1987). These cross peaks show splitting of the basic pattern (i.e., no coupling to a third spin) into two identical patterns displaced by the passive coupling of the common coupling partner in the respective dimension. Even if one of these coupling constants is smaller than the line width, it can still be determined to high degree of accuracy by cross sections taken through the components of the appropriate cross peak in ω_2 axis (discussed below).

Once three mutually coupled protons are identified, individual three spin systems can be assigned to the appropriate residue by linking these resonances to the rest of the spin system. For sugars having the β-galacto configuration, this can be achieved by interresidue NOE (Figure 1c) connectivities observed between H-1 and H-5 resonances. Theoretically due to the equatorial nature of the H-4 resonance, NOE connectivity from H-4 to H-5 can also be used regardless of the anomeric configuration of galactopyranoses. However, in practice these connectivities are often obscured by overlap with other resonances (or the diagonal) in crowded regions of the NOESY spectrum. For α-galactopyranoses, connectivity can be obtained from the HMBC spectrum (discussed below), in which anomeric proton resonances show long-range correlation to the C-5 resonance (then to H-5 by HMQC) of the same residue.

The method outlined above was used to assign proton resonances of individual residues in the repeating unit. The resonance at 5.218 ppm, which belongs to one of the β-Gal$_f$ residues (residue A), shows cross peak connectivity up to H-3 in the DQF-COSY spectrum. The H-2/H-3 cross peak shows a distorted multiplet structure, indicating strong coupling between the H-3 and H-4 resonances. A cross section taken through H1 in the HOHAHA spectrum also shows H-2 and a weak peak for H-3. The anomeric resonance (5.071 ppm) of the remaining β-Gal$_f$ residue (residue C) shows correlation of H-2 in the DQF-COSY spectrum. The HOHAHA spectrum shows H2 and a weak cross peak to H-3 from H-1, indicating that the H-2 and H-3 resonances have close chemical shifts. The H-2/H-3 cross peak, which partially overlaps with the diagonal in the DQF-COSY spectrum, can be seen in the expansion of the E-COSY spectrum due to the narrow diagonal peaks in the latter spectrum (Figure 2b). The H-3/H-4 cross peak shows overlap with other cross peaks in both DQF and E-COSY spectra, preventing assignment of the H-4 resonance. The H-1/H-2 cross peak of this residue shows a characteristic E-COSY multiplet pattern, indicating a four-bond coupling between H-1 and H-3 protons. This rather unusual long-range coupling ($J_{1,3}$ = 1.0 Hz) could be attributed

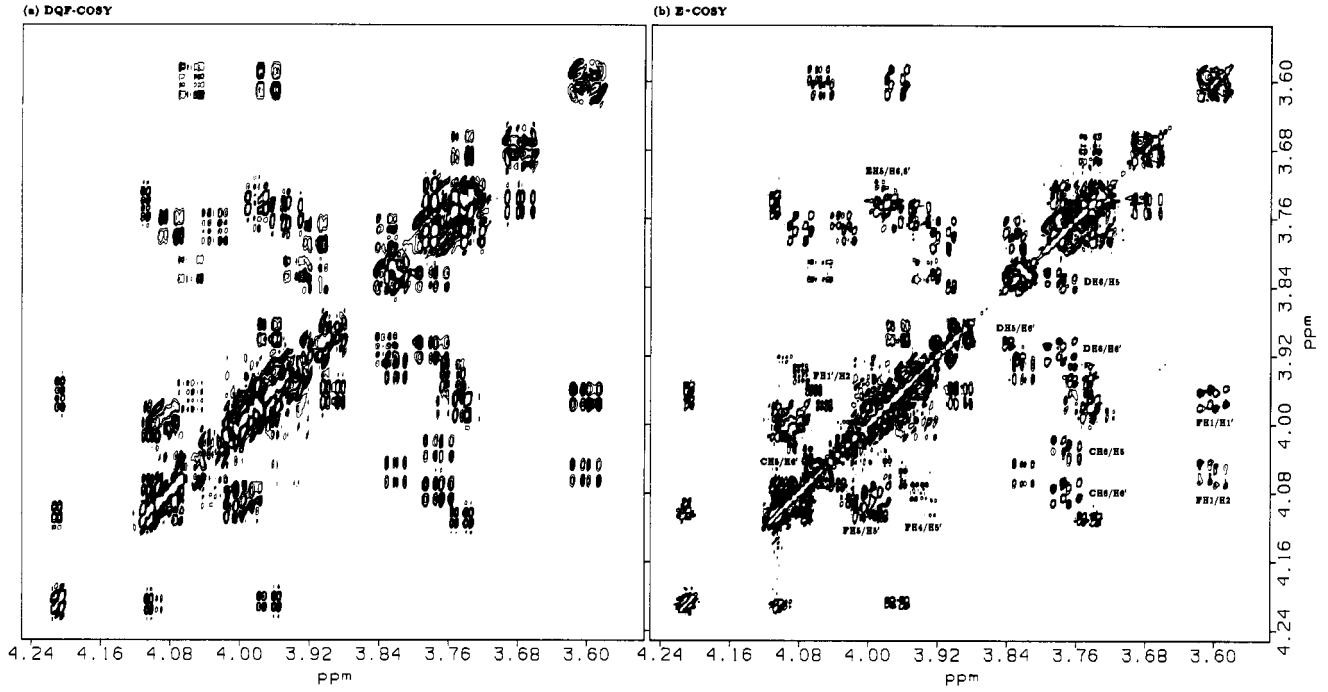

FIGURE 2: Expanded regions of (a) the DQF-COSY spectrum and (b) the E-COSY spectrum of the *S. oralis* C104 polysaccharide at 600 MHz. The data matrix for the E-COSY spectrum was 710 × 4K (TPPI) complex points with 32 scans per t_1 value. Acquisition and processing parameters are identical with those of Figure 1a. Both spectra have digitial resolutions of ±0.0018 ppm/point. Cross peaks involving three mutually coupled spins are labeled in the figure (see the text for notation). Positive contours are drawn dark.

to the unique "W" arrangement between these protons in β-Gal$_f$ residue. This coupling was confirmed by a weak H-1/H-3 cross peak that was seen in the lower contours of the E-COSY spectrum. Although this effect is also seen in the H-1/H-2 cross peaks of the other Gal$_f$ residue (A), $J_{1,3}$ could not be measured due to distortion caused by the strong coupling between the H-3 and H-4 resonances. Unlike galactopyranose residues, where HOHAHA spectra yield strong cross peaks for H-2, H-3, and H-4 from the anomeric protons, galactofuranose residues show cross peaks to H-2 with medium intensity and a weak peak to H-3. This is due to the small coupling between H-1/H-2 ($J_{1,2} < 2$ Hz) and H-1/H-3 ($J_{1,3} \leq 1.0$ Hz). Even though the coupling constants between other vicinally coupled protons in these residues are larger than $J_{2,3}$, poor chemical shift dispersion among these resonances (see Table I) prevented the assignments by homonuclear correlation spectra. However, once partial assignments are made, heteronuclear correlation spectra, particularly HMQC-COSY (discussed below), can be used to obtain complete ^{1}H and ^{13}C assignments of the residues, including strongly coupled protons.

The anomeric resonance at 4.959 ppm ($J_{1,2}$ 3.6 Hz), which belongs to the α-Gal (residue E), shows connectivity up to H-4 in both the COSY and in the HOHAHA spectrum. The β-anomeric resonance at 4.635 ppm ($J_{1,2}$ 8.4 Hz) gave a cross peak to the resonance at 3.942 ppm (H-2), which in turn shows its directly bonded carbon atom at 53.31 ppm (C-2) in the HMQC spectrum (Figure 3), indicating that this residue is *N*-acetylgalactosamine (residue D). Both DQF-COSY as well as HOHAHA spectra show identical chemical shift values for the H-2 and H-4 resonances. The remaining anomeric resonance (4.507 ppm, $J_{1,2} = 8.0$ Hz), which belongs to β-galactose (residue B), gave connectivity up to H-4 in DQF-COSY and also in HOHAHA spectra.

The expanded region of the E-COSY spectrum in Figure 2b shows five sets of cross peaks each belonging to three mutually coupled spins. One such system involves H-5, H-6, and H-6′ resonances of the β-GalNAc (D) residue. H-5 was assigned by interresidue NOE to H-1 (Figure 1c and Table II). Because the passive spin is unperturbed by the mixing process in E-COSY, cross peak multiplets for a three-spin system can be imagined as arising from a superposition of displaced two-spin multiplets. Therefore, the weakly coupled H-5, H-6, and H-6′ spin system in a galactopyranose residue give the simplest E-COSY pattern due to vanishing $J_{4,5}$ (<1 Hz), i.e., each cross peak contains two ($\frac{+}{-}_{+}$) square patterns displaced by couplings to the passive spin. Coupling constants and their signs can be obtained from the cross peaks as described elsewhere (Griensinger et al., 1987). For example, E-COSY cross peaks of β-GalNAc from both sides of the diagonal gave chemical shift assignments of the protons (H-5, −3.829; H-6, −3.775; and H-6′, −3.911 ppm) and also the sign and magnitude of the coupling constants involved ($J_{5,6} = 7.7$ $J_{5,6'} = 3.6$, $J_{6,6'} = -10.9$ Hz). These values were obtained as passive couplings in the cross peaks by examining ω_2 cross sections taken through the cross-peak components.

For a three-spin system with nonvanishing couplings to a fourth resonance, vicinal cross peaks show splitting of the basic four-component pattern into eight components. For instance, H-5/H-6 and H-5/H-6′ cross peaks of galactofuranose show such a pattern due to the splitting of the H-5 signal by $J_{4,5}$. This can be used to distinguish methylene protons and their coupled partners of galactopyranoses from those of other sugars including alditols. The most upfield proton resonance (3.599 ppm) shows the characteristic E-COSY cross peak with large active coupling ($J = -10.8$ Hz) to the resonance at 3.962 ppm identifying these two resonances as methylene protons. Both protons show cross peaks to their mutually coupled partner at 4.052 ppm. This resonance (H-2) in turn extends the assignments to two more vicinal protons (H-3 and H-4), indicating that the spin system belongs to the ribitol residue (residue F) in the repeating unit. Numbering of resonances in ribitol is according to C-5 being involved in the phosphodiester linkage. Since the methylene carbon at 69.42 ppm (attached methylene protons at 3.965 and 3.599 ppm) does not show any ^{31}P coupling, it was identified as C-1 of ribitol. Even though the cross peaks for the H-4, H-5, and H-5′

Table I: NMR Chemical Shifts of the Polysaccharide from *S. oralis* C104 in D_2O at 25 °C[a]

	residue					
assignment	β-Gal$_f$ A	β-Gal B	β-Gal$_f$ C	β-GalNAc D	α-Gal E	ribitol F
^1H (ppm)						
H-1	5.218	4.507	5.071	4.635	4.959	3.599
H-1′						3.965
H-2	4.208	3.675	4.068	3.944	3.890	4.054
H-3	4.101	3.742	4.100	3.752	3.963	3.818
H-4	4.108	4.107	4.006	3.945	4.204	3.933
H-5	3.990	3.730	4.027	3.829	3.980	3.990 (5.5)[b]
H-5′						4.085 (5.5)[b]
H-6	3.97[c]	3.78[c]	3.769	3.775	3.74[c]	
H-6′	3.97[c]	3.78[c]	4.077	3.911	3.74[c]	
NAc				2.040		
J_{HH} (Hz)						
$J_{1,1'}$						−10.8
$J_{1,2}$	1.8	8.0	1.8	8.4	3.6	7.2
$J_{1,3}$	*d*		1.0			
$J_{1',2}$						2.7
$J_{2,3}$	*d*	10.0	3.4	10.8	10.4	5.8
$J_{3,4}$	*d*	3.2	6.2	3.3	3.2	7.1
$J_{4,5}$	*d*	*e*	4.3	*e*	*e*	7.0
$J_{4,5'}$						<2.8
$J_{5,5'}$						−10.8
$J_{5,6}$	*d*	*d*	7.3	7.7	*d*	
$J_{5,6'}$	*d*	*d*	3.7	3.6	*d*	
$J_{6,6'}$	*d*	*d*	−10.9	−10.7	*d*	
^{13}C (ppm)						
C-1	110.05	103.91	108.62	103.93	99.92	69.48
$^1J_{CH}$	(175)[f]	(162)	(174)	(162)	(171)	
C-2	82.26	70.84	81.81	53.36	68.26	71.63
C-3	77.46	81.09	77.51	71.56	80.22	72.29
C-4	83.36	69.38	83.96	68.59	70.08	71.66 (7.1)[b]
C-5	70.10 (10.1)[b]	75.96	70.38	74.54	71.43	67.40 (5.6)[b]
C-6	67.18 (6.4)[b]	61.85	71.91	68.08	62.06	
NAc						
CH_3				23.13		
CO				176.00		

[a] Chemical shifts are with reference to internal acetone (^1H, 2.225 ppm; ^{13}C, 31.07 ppm) and external 85% H_3PO_4 (^{31}P, 0 ppm). Coupling constant values are in hertz (error approximately ±1 Hz). For three mutually coupled protons, values were measured as passive coupling constants from E-COSY. Other values were measured from splitting in DQF or E-COSY cross peaks as active couplings and were uncorrected for line width effects. [b] J_{PH} coupling constants were from E-COSY multiplets whereas J_{PC} coupling constants were from resolution-enhanced ^{13}C spectrum. [c] Strongly coupled methylene protons. [d] Coupling constants could not be extracted from COSY multiplets due to strong coupling. [e] Not observed. Generally less than 1 Hz. [f] Anomeric $^1J_{CH}$ (±2.5 Hz) coupling constants are from HMQC-COSY spectrum. Other carbons except methyl carbons show one-bond C–H couplings of 151–140 Hz.

protons of this residue are seen in the E-COSY spectrum, neither coupling constants nor the chemical shift values for H5 and H5′ protons could be extracted from the spectra due to distortion of cross peaks resulting from ^{31}P couplings to these protons. The E-COSY spectrum also shows another set of three mutually coupled spins (H6′, −4.077; H6, −3.769; H-5, −4.027 ppm; and $J_{5,6}$ = 7.3, $J_{5,6'}$ = 3.7, $J_{6,6'}$ = −10.9 Hz) with $J_{4,5}$ = 4.3 Hz, indicating that the resonances belong to one of the galactofuranose residues. Other methylene protons in the molecule could not be assigned in the E-COSY spectrum due to strong couplings between the resonances.

The ^{13}C spectrum of the *S. oralis* C104 polysaccharide (data not shown) shows five anomeric resonances at 110.05, 108.62, 103.93, 103.91, and 99.92 ppm. It also shows characteristic resonances for an acetamido sugar, including a resonance at 53.36 ppm for a ring carbon atom to which a NAc group is attached, a methyl carbon at 23.13 ppm, and a carbonyl carbon of the NAc group at 176.00 ppm. The ^{13}C DEPT experiment (Doddrell et al., 1982) shows seven methylene carbons (see Figure 4), and two of them show ^{31}P couplings (67.40 ppm, J_{PC} = 5.6 Hz, and 67.18 ppm, J_{PC} = 6.4 Hz) indicating a phosphodiester linkage between two methylene carbon atoms.

Correlation peaks observed in the HMQC spectrum were used to assign carbon resonances by direct correlation to attached carbons. The anomeric carbon resonances at 110.05 and 108.62 ppm show correlation to proton resonances at 5.218 and 5.071 ppm, indicating that the both Gal$_f$ residues have the β-anomeric configuration (Beier et al., 1980). The other anomeric carbons were assigned by direct correlation to their attached protons. However, some correlation peaks could not be identified due to incomplete proton assignments, overlap of correlation peaks and difficulties in identifying ^{13}C resonances from protons for which the chemical shift separation is less than the digital resolution of the ω_2 axis. It has been shown before (Abeygunawardana et al., 1990) that some of these ambiguities can be overcome by the use of long-range correlations in HMBC spectra. This method is ideally suited for hexopyranoses, where long-range correlations can be predicted on the basis of known long-range coupling constants (Morat et al., 1988) resulting from fixed ring geometry. However, this is of limited used in assigning carbon resonances of the galactofuranoses and the ribitol residue in the C104 polysaccharide since long-range ^1H–^{13}C coupling constants of furanoses and the ribitol residue in the polysaccharide are not known. The HMQC-COSY experiment, which utilizes the advantageous feature of greater spectral dispersion in ^{13}C dimension of the HMQC experiment, combined with COSY provide an alternative method for assignments. Since galactofuranose residues contain medium to large vicinal couplings in ^1H spectra except for $J_{1,2}$ < 2 Hz (Richards et al., 1989;

Table II: Summary of Observed Connectivities in NMR Spectra of *S. oralis* C104 Polysaccharide

expt	β-Gal$_f$ A[a]	β-Gal B	β-Gal$_f$ C	β-GalNAc D	α-Gal E	ribitol F
E-COSY[b]						
H-1	H-2,3	H-2	H-2,3	H-2	H-2	H-1′,2
H-5			H-6,6′	H-6,6′	H-6,6′	H-4,5′
HOHAHA						
H-1	H-2,3	H-2,3,4	H-2,3	H-2,3,4	H-2,3,4	H-1′,2,3,4
NOESY						
H-1	H-2,3	H-3,5	H-2	H-3,5	H-2	H-1′
	BH-3[c]	CH-6′[c]	DH-6[c]	EH-3[c]	FH-1′,1[c]	EH-1[c]
HMQC-COSY[d]						
C-1	H-2(w)	H-2	H-2(w)	H-2	H-2	
C-2	H-1,3	H-1,3	H-1,3	H-1,3		
C-3	H-2,4	H-2,4	H-2,4	H-2,4	H-2,4	
C-4	H-3	H-3	H-3,5	H-3		
C-5	H-6,6′	H-6,6′	H-4,6,6′	H-6,6′		
C-6	H-5	H-5	H-5	H-5		
HMBC						
H-1	A4, A3(w), B3[c]	C6[c]	C4, C3(w), D6[c]	E3[c]	E3, E5, F1[c]	F2, E1[c]
H-1′						E1(w)[c]
H-2	A3(w), A1	B3, B1	C3(w), C1	D1, D3	E3	F1(w)
H-3	A2(w), A5(w)	B2, A1[c]	C2(w), C5(w)	D2(w)	E2	F1, F2, F4, F5
H-4	A3(w), A6(w)	B3, B2	C3(w)	D3, D2	E3, E2	
H-5		B4, B6(w)				
H-6		B4(w), B5	C5	D5, D4(w), C1[c]	E4(w)	
H-6′				C1[c]		

[a] The individual residues are identified by capital letters, and the ^{13}C resonance is indicated by a capital letter followed by a number for the carbon atom assigned. [b] DQF-COSY and E-COSY show cross peaks to vicinal and geminal protons where $J_{active} > 1$ Hz. However, some connectivities could not be obtained due to strong couplings or cross-peak overlap. [c] Interresidue connectivities. [d] All protons shows signals arising from one-bond correlations with their attached carbon atoms in HMQC as well as HMQC-COSY (w = weak peak).

Sznaidman et al., 1986), chemical shift overlap in the COSY spectra can be resolved by HMQC-COSY spectra recorded without ^{13}C decoupling during the acquistion (Abeygunawardana et al., 1991). In this experiment a cross section taken through a particular carbon frequency shows a direct correlation peak split by $^1J_{CH}$ coupling that is analogous to the diagonal peak in the COSY spectru, in addition to relay peaks to vicinal protons. If vicinal protons are strongly coupled or closely spaced, relay peaks in HMQC-COSY appear between the components of the direct peak (Figure 4), thereby allowing accurate assignments. Large vicinal proton couplings give stronger relay peaks whereas smaller couplings tend to give weaker peaks.

The utility of this method can be demonstrated in assigning proton and carbon resonances in furanoid residues. C-2 resonances of both residues can be identified by relay peaks to corresponding H-1 resonances. Figure 4, which is an expansion of a crowded region in the HMQC-COSY spectrum, illustrates connectivity observed for residue C. Each vicinal proton pair shows a square pattern of connectivity, i.e., relay peaks from one carbon connecting to the middle of the direct peaks for adjacent carbon and vice versa. Connectivity from C2 (C-2 of residue C) shows overlap of C3 with A3.

Since relay peaks to vicinal protons also have the same ^{13}C chemical shift labeling as the direct peak, resolved relay peaks can be used to obtain assignments of overlapping direct peaks. Relay peaks from overlapping C3 and A3 indicated the slightly different ^{13}C chemical shifts for these resonances and accurate values were then obtained from the normal ^{13}C spectrum. The relay peak C3/H4 connects to a resonance at 83.91 ppm, indicating it as C4. Likewise, connectivity can be extended for all the resonances in the residue including H6, H6′, and the methylene carbon. These methylene protons together with H-5 gave chemical shifts identical with one of the three spin systems identified from the E-COSY spectrum. As expected, C5 gave a weak relay peak to H6′($J_{5,6'}$ = 3.7 Hz). Similarly, relay peaks observed in A3 extend the assignments to A4. A3/H4 and A4/H3 peaks gave accurate assignments for H-3 and H-4, which were otherwise strongly coupled in homonuclear spectra. The much weaker A4/H5 relay peak showed slight overlap with an intense direct component of A4. This prevented extending the assignment to A5 and A6. Since methylene carbons are identified by DEPT spectra, correlations observed in HMQC can be used to obtain chemical shift assignments of methylene carbons. Galactopyranose residues often show relatively large vicinal couplings between H-5 and H-6 protons. Therefore, relay peaks from the methylene carbon resonance can identify the proton attached to the adjacent carbon atom. For example, the methylene carbon at 68.08 ppm in HMQC-COSY shows a relay peak to a proton that was previously assigned as β-GalNAc H5. D5 also shows relay peaks to both H6 protons. As expected, the relay peak arising from the small $J_{4,5}$ was not seen in the spectrum. In the HMQC spectrum, the D6′ peak shows overlap with another correlation peak, which in HMQC-COSY shows relay peaks to α-Gal H-1 and H-3, thereby identifying it as E-2.

This approach can be used to assign the remaining methylene carbons and their attached protons. The methylene carbon at 61.96 ppm gave a broad correlation peak in HMQC, indicating strong coupling between geminal protons. The relay peak from this carbon showed a proton resonance at 3.973 ppm and its directly bonded carbon at 71.39 ppm. This carbon resonance was identified as E-5 on the basis of long-range correlation from H-1 of α-Gal residue in the HMBC spectrum (Figure 5).

The methylene carbon at 61.79 ppm, which also showed strongly coupled attached protons gave a relay peak at 3.725 ppm. This proton was identified as H-5 of residue B by long-range correlation to B4 in the HMBC spectrum. This assignment is also consistent with the broad unresolved cross peak (H-3, H-5) observed in the NOESY spectrum from H-2. The methylene carbon at 69.42 ppm was assigned as ribitol C-1 by direct correlation to its attached protons.

The methylene carbon resonance at 67.40 ppm, which showed ^{31}P–^{13}C couplings (J_{PC} = 5.6 Hz), was identified as F5 on the basis of long-range couplings to H-3 of ribitol. In

Table III: Structures of the Receptor Polysaccharides from Streptococcal Strains That Participate in Lectin-Mediated Coaggregation with *Actinomyces* spp[a]

S. mitis J22 Polysaccharide

$$[\rightarrow 6)Gal_pNAc(\alpha 1\rightarrow 3)Rha_p(\beta 1\rightarrow 4)Glc_p(\beta 1\rightarrow 6)Gal_f(\beta 1\rightarrow 6)\underline{Gal_p(\beta 1\rightarrow 3)Gal_pNAc}(\alpha 1\rightarrow PO_4^-)]_n$$
$$\nearrow$$
$$Rha(\alpha 1\rightarrow 2)$$

S. oralis ATCC 10557 De-*O*-acetylated Polysaccharide

$$[\rightarrow 6)Gal_p(\alpha 1\rightarrow 3)Rha_p(\beta 1\rightarrow 4)Glc_p(\beta 1\rightarrow 3)Gal_f(\beta 1\rightarrow 6)\underline{Gal_p(\beta 1\rightarrow 3)Gal_pNAc}(\alpha 1\rightarrow PO_4^-)]_n$$

S. oralis 34 Polysaccharide

$$[\rightarrow 6)Gal_pNAc(\alpha 1\rightarrow 3)Rha_p(\beta 1\rightarrow 4)Glc_p(\beta 1\rightarrow 6)Gal_f(\beta 1\rightarrow 6)\underline{Gal_pNAc(\beta 1\rightarrow 3)Gal_p}(\alpha 1\rightarrow PO_4^-)]_n$$

S. oralis C104 Polysaccharide

$$[\rightarrow 6)Gal_f(\beta 1\rightarrow 3)Gal_p(\beta 1\rightarrow 6)Gal_f(\beta 1\rightarrow 6)\underline{Gal_pNAc(\beta 1\rightarrow 3)Gal_p}(\alpha 1\rightarrow 1)ribitol(5\rightarrow PO_4^-)]_n$$

[a] Underlined disaccharides represent the proposed lectin receptor regions of these molecules.

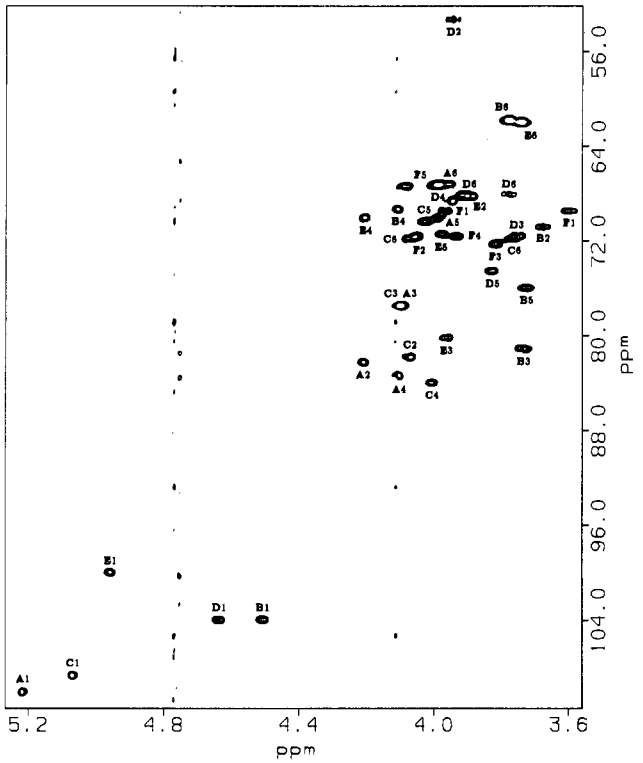

FIGURE 3: Phase-sensitive, ^{13}C-decoupled, ^1H-detected multiple-quantum correlation spectrum ($^1H[^{13}C]$HMQC) of the polysaccharide from *S. oralis* C104 at 600 MHz. The data matrix was 256 × 2K (TPPI) complex points with 32 scans per t_1 value. The spectral window was ±5555 Hz in the F_1 dimension (^{13}C) and ±1046 Hz in the F_2 dimension (^1H). Relaxation delays of 3 s and 3.45 ms, ($1/2^1J_{CH}$) and a 495-ms acquisition time were used in the experiment. Data were apodized in t_2 by Gaussian line broadening of 4 Hz and cosine bell in t_1 and zero filled in both dimensions to obtain a 1K × 2K real matrix with a digital resolution of ±0.0017 ppm/point (^1H) and ±0.07 ppm/point (^{13}C). Peaks are labeled with a capital letter identifying the residue followed by the number assigning the carbon atom.

the HMQC spectrum, H-5 of this residue showed an overlapping correlation peak with unresolved methylene proton resonances of the remaining methylene carbon (A6, 67.17 ppm), which also showed ^{31}P–^{13}C couplings (J_{PC} = 6.4 Hz) indicating a phosphate linkage. A6 showed a relay peak to H5 at 3.990 ppm, which is directly correlated with the carbon resonance at 70.08 ppm also split by ^{31}P–^{13}C couplings (J_{PC} = 10.1 Hz). These assignments were supported by weak long-range correlations (A6/H4) observed in the HMBC spectrum. Since these assignments essentially completed the proton assignments in the repeating unit, the rest of the carbon assignments can be obtained by direct correlation to their attached protons in the HMQC at 600 MHz. Complete proton and carbon assignments are summarized in Table I.

Linkage assignments between the residues in the repeating unit were obtained directly from the observed long-range correlations in the HMBC spectrum. All the anomeric protons in the polymer showed long-range correlation to the aglycon carbon atom in the adjacent residue defining the glycosidic linkage. The anomeric proton of residue A showed long-range correlation to A4, A3, and B3, defining the linkage as Gal_f-($\beta 1\rightarrow 3$)Gal. H-1 of residue B gave correlation to C6 as the only long-range connectivity in HMBC, indicating a Gal-($\beta 1\rightarrow 6$)Gal_f linkage. The anomeric proton of residue C showed linkage across the glycosidic bond to D6 in addition to C3 and C4. Residue D as in the case of the other β-Gal residue gave long-range correlation only to E3, defining the GalNAc($\beta 1\rightarrow 3$)Gal α-linkage. α-GalNAc H-1 showed correlations to E3, E5, and also to F1, indicating a Gal($\alpha 1\rightarrow$-1)ribitol linkage. These linkage assignments yield the following structure for the repeating unit:

$$[\rightarrow 6)\underset{A}{Gal_f}(\beta 1\rightarrow 3)\underset{B}{Gal_p}(\beta 1\rightarrow 6)\underset{C}{Gal_f}(\beta 1\rightarrow 6)\underset{D}{DGal_pNAc}(\beta 1\rightarrow 3)\underset{E}{Gal_p}(\alpha 1\rightarrow$$
$$\underset{F}{1)ribitol(5\rightarrow PO_4^-)}]_n$$

Long-range correlations together with observed connectivities in other NMR spectra are summarized in Table II. ^{31}P couplings observed in A6, A5, F4, and F5 support the chemical evidence of phosphodiester linkage between methylene carbons of ribitol and galactose residues.

Discussion

On the basis of the determination of its complete structure, the cell surface polysaccharide of *S. oralis* C104 recognized by the lectins of *A. naeslundii* WVU45 (ATCC 12104) and *Bauhinea purpurea* has been identified as a ribitol teichoic acid. Similar molecules containing glycosyl units linked by ribitol phosphate have previously been found among the type specific antigens of *Streptococcus pneumoniae*, a close relative of *S. oralis* (Kilpper-Balz et al., 1985) and also among the capsular antigens of certain gram negative bacteria such as *Haemophilus influenzae* (Kenne & Lindberg, 1983). The presence of ribitol phosphate in the C104 polymer represents an important structural difference between the receptor molecule of this strain and those of three previously studied strains (Abeygunawardana et al., 1989, 1990, 1991), each of

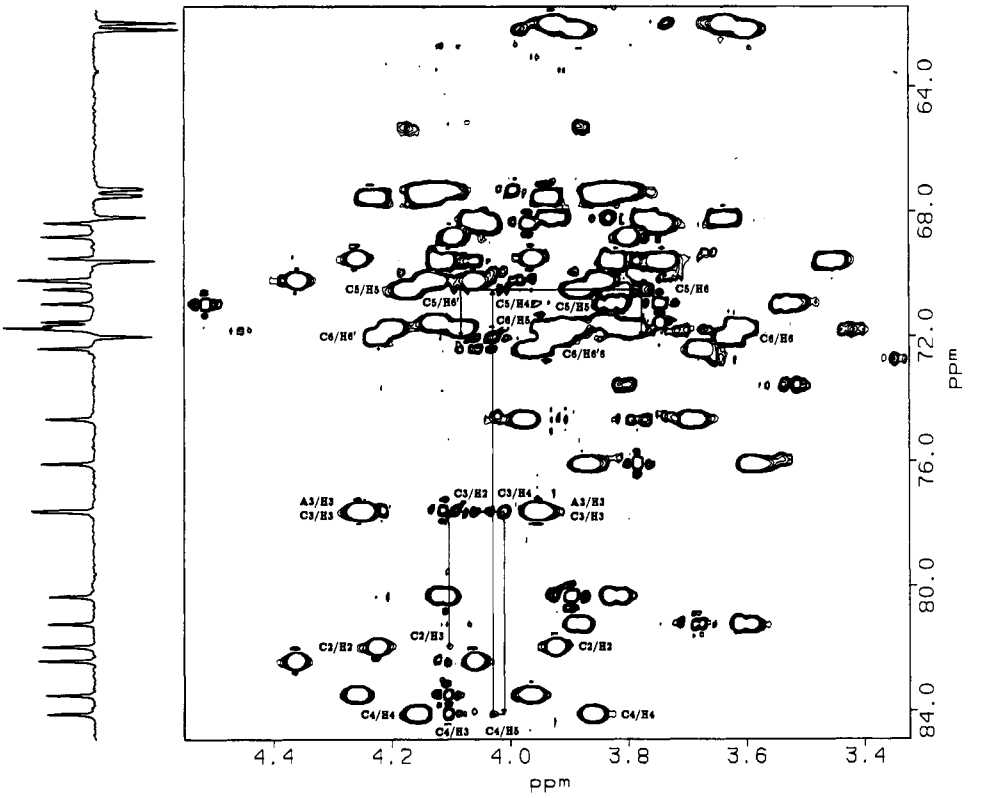

FIGURE 4: Phase-sensitive HMQC-COSY spectrum of the polysaccharide at 500 MHz recorded without ^{13}C decoupling during the acquisition. The contour map shows the most crowded region of the spectrum with the square pattern indicating relay connectivities for residue C. Peaks are labeled as discussed in the text. The data matrix was 2 × 256 × 1K complex points with 128 scans per t_1 value. The spectral window was ±6250 Hz in the F_1 dimension and ±1200 Hz in the F_2 dimension. A relaxation delay of 1.5 s was used. Gaussian line broadening (2 Hz) in t_2 and cosine bell apodization in t_1 were used prior to Fourier transformation. Data were zero filled in the t_1 dimension to obtain 1K × 1K real matrix with a digital resolution of ±0.005 ppm/point (^1H) and ±0.1 ppm/point (^{13}C). Expansion of the ^{13}C DEPT spectrum recorded with a 135° flip angle (^1H) at 125 MHz is displayed at the left.

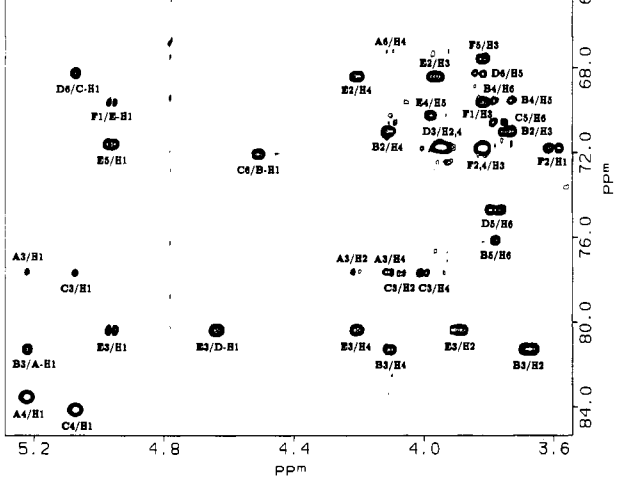

FIGURE 5: ^1H-detected, ^1H–^{13}C multiple-bond correlation spectrum ($^1H[^{13}C]HMBC$) of the polysaccharide at 500 MHz. The data matrix was 2 × 256 × 1K complex points with 32 scans per t_1 value. The spectral window was ±6250 Hz in F_1 dimension and ±1024 Hz in F_2. Relaxation delays of 1.5, 3.4, ($1/2\ ^1J_{CH}$), and 50 ms ($1/2^nJ_{CH}$) and a 500-ms acquisition time were used in the experiment. Data were apodized in t_2 by Gaussian line broadening of 3 Hz and a cosine bell in t_1 and zero filled to obtain a 1K × 1K real matrix with a digital resolution of ±0.004 ppm/point (^1H) and ±0.1 ppm/point (^{13}C). The data are presented in the mixed mode (Bax & Marion, 1988), absorption in F_1 and absolute value in F_2.

which contains a phosphodiester linkage involving a glycosyl phosphate residue. (See Table III for comparison of the structures of the polysaccharide receptors in this series.)

Another notably different property of the C104 polysaccharide is the stability of the polymer at low pH (2.0), under which condition the other three polysaccharides in Table III show partial depolymerization by cleavage of glycosyl phosphate bond. *S. oralis* C104 polysaccharide also showed sharper lines in the NMR spectra than the others and gave weaker NOE cross peaks in the NOESY spectra at 25 °C for similar mixing times, indicating a high degree of internal motion associated with the repeating structure. This flexibility is presumably due to the ribitol and the additional 6-linked galactofuranose residue in the repeating unit. The biological significance of these differences remains to be explored.

The presence of GalNAc(β1→3)Galα in the *S. oralis* C104 repeating unit strongly supports the proposal that this structure and Gal(β1→3)GalNAcα linked by Gal$_f$(β1→6) linkage represents a highly conserved receptor region recognized by the type 2 fimbrial lectin of *A. vicosus* and *A. naeslundii* (Abeygunawardana et al., 1991). Moreover, the absence of cross reactivity between the *S. oralis* C104 and 34 polysaccharides each containing Gal$_f$(β1→6)Gal$_p$NAc(β1→3)-Gal$_p\alpha$ is consistent with the hypothesis that the lectin receptor regions of these molecules are relatively nonimmunogenic, presumably because internal GalNAc(β1→3)Gal is exposed in a way that mimics the termini of host cell molecules such as globosides (McIntire et al., 1988). In spite of the structural similarity of their receptor molecules, *S. oralis* 34 participates in lectin-mediated coaggregations with strains of *A. viscosus* and *A. naeslundii* while *S. oralis* C104 exhibits a preference for certain strains of the latter species (Cisar et al., 1979). It remains to be determined whether the receptor polysaccharides of other streptococcal strains that coaggregate selectively with the latter group of *A. naeslundii* strains (Kolenbrander &

Williams, 1983) also are of the ribitol teichoic acid type. Clearly, the application of high-resolution NMR is a highly feasible approach to characterize this type of polysaccharide.

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Registry No. *Streptococcus oralis* C104, 135106-68-2.

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