Structure of a Streptococcal Adhesin Carbohydrate Receptor*

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Interactions between complementary protein and carbohydrate structures on different genera of human oral bacteria have been implicated in the formation of dental plaque. The carbohydrate receptor on Streptococcus sanguis H1 (one of the primary colonizing species that is specific for the adhesin on Capnocytophaga ochracea ATCC 33596 (a secondary colonizer) has been isolated from the streptococcal cell wall, purified, and structurally characterized. The hexasaccharide repeating unit of the polysaccharide was purified by reverse-phase, amino-bonded silica, and gel permeation chromatography. The 1H and 13C shift data were established that the repeating unit was a hexasaccharide composed of rhamnose, galactose, and glucose in the ratio of 2:3:1, respectively. In the present study, determination of absolute configuration by gas chromatography of the trimethylsilyl (+)-2-butyl glycosides revealed that the rhamnose residues were of the L configuration while the hexoses were all D. The heteronuclear multiple bond correlation experiment. These data show that the structure of the hexasaccharide repeating unit derived from the cell wall polysaccharide of S. sanguis H1 is: α-L-Rhap-(1→2)-α-L-Rhap-(1→4)-α-L-Rhap-(1→3)-β-D-Galp-(1→2)-β-D-Galp-(1→4)-β-D-Galp-(1→3)-α-L-Rhap.

Protein-carbohydrate interactions appear to be necessary for initial attachment and in the infection process of viruses (1, 2), mycoplasma (3, 4), protozoas (5–7), and bacteria (8–10). Receptors for microbial carbohydrate-binding proteins have been found on glycolipids (10, 11), glycoproteins (12–14), and on bacterial polysaccharides (15–17). With a more complete understanding of the molecular mediators involved in attachment, therapeutics may be designed to effectively interrupt the process and obviate the attachment altogether (18, 19).

In the human oral environment, a tremendous diversity of microbes interact in a complex ecosystem. This diversity is manifested in dental plaque, consisting of numerous microbes, salivary components, and extracellular bacterial polymers. Intergeneric coaggregation (specific bacterial interactions dependent on adhesin to carbohydrate binding) appears to play a role in the establishment and maturation of dental plaque (19, 20). Coaggregation may occur between genetically unrelated Gram-positive, Gram-negative, or between Gram-negative and Gram-positive partners. Many of these coaggregations are inhibitable in vitro by simple sugars such as lactose, L-rhamnose (Rha), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid. Of the bacteria found in dental plaque, Streptococcus sanguis, Actinomyces viscosus, and Actinomyces naeslundii are the predominant primary colonizers and each is prevalent in mature dental plaque.

Three bacterial polysaccharides that appear to act as adhesin receptors have been studied in detail to date. S. sanguis strain H1 coaggregates with Capnocytophaga ochracea ATCC 33596 (16), while both S. sanguis strain 34 and S. sanguis strain H1 coaggregates with Capnocytophaga ochracea ATCC 33596 (16), while both S. sanguis strain 34 and S. sanguis 1. The abbreviations used are: Rha, L-rhamnose; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; GC/MSD, gas chromatography with mass-selective detection; GLC, gas-liquid chromatography; PDMS, 25C plasma desorption mass spectrometry; COSY, two-dimensional \((^1H,^1H)\) shift correlation spectroscopy; f.d., free-induction decays; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; TPEI, time-proportional phase incrementing; HETCOR, two-dimensional \((^1C,^1H)\) shift correlation spectroscopy; HMBC, heteronuclear multiple quantum coherence spectroscopy; FAB, fast atom bombardment.

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Streptococcal Adhesin Carbohydrate Receptor

strain J22 are responsible for the coaggregation with A. viscosus T14V (15, 17). The former mediates an interaction between Gram-positive and Gram-negative cell types, while the latter two are responsible for the interaction between two Gram-positive partners. The *S. sanguis* 34 coaggregation carbohydrate receptor moiety is a cell wall polysaccharide consisting of hexasaccharide repeating units with intrachain phosphodiester linkages (15, 21). The *S. sanguis* J22 receptor is a cell wall polysaccharide composed of a heptasaccharide subunit linked by phosphodiester bonds (17). The coaggregation polysaccharide from *S. sanguis* H1 is also a cell wall polysaccharide, consisting of monophosphorylated hexasaccharide repeating units (16). The glycosyl residues that constitute each respective repeating unit vary, with Rha, Gal, Glc, and GalNac present in a ratio of 1:2:1:2 in *S. sanguis* 34, Rha, Gal, Glc, and GalNac (2:2:2:2) in *S. sanguis* J22, and Rha, Gal, and Glc (2:3:1) in *S. sanguis* H1. The present study describes the structural characterization of the hexasaccharide repeating unit from the coaggregation polysaccharide of *S. sanguis* H1.

**EXPERIMENTAL PROCEDURES**

**Bacterial Culture Conditions—** *S. sanguis* H1 and *C. ochracea* ATCC 35508 were obtained from Dr. P. Cohenbrander (National Institute of Dental Research). All cells were grown under anaerobic conditions. *S. sanguis* H1 was grown in a complex medium containing Tryptone, yeast extract, Tween 80, and K2HPO4, with 0.5 mM glucose (22), and *C. ochracea* ATCC 35508 was cultivated in Schneider broths (BBL Microbiology Systems).

**Hexasaccharide Purification—** A modification of an earlier procedure (16) giving increased yield of purified hexasaccharide was utilized. Briefly, intact *S. sanguis* H1 cells were sequentially treated with 0.1% Triton X-100, 0.1% Pronase (Calbiochem), and 6 M guanidine HCl, with extensive washing after each treatment. This crude cell wall preparation was then digested by incubation with 20 mg/ml corresponding to 4 mg/g initial wet weight *S. sanguis* H1 cells, and those fractions containing visible residue were spotted on high performance thin-layer chromatography (HPTLC) plates. Hexasaccharide-containing fractions were pooled, evaporated, suspended in a mixture of acetonitrile and water (0.5 ml), and applied to an 8 mm × 30 cm MicroPak AX-5 Varian dimethylpropyl-silica HPLC column (25). The hexasaccharide was eluted by passing a linear gradient of methanol (0.5 ml/min) from a Supelco column (Supelco) in high performance liquid chromatography (HPLC) grade water are expressed in parts/million downfield from internal DSS, with an accuracy of 0.002 ppm.

**Nuclear Magnetic Resonance Spectroscopy—** A sample (14 mg) of the hexasaccharide repeating unit of the cell wall polysaccharide from *S. sanguis* H1 was subjected to NMR spectroscopic analysis. The sample was repeatedly dissolved in D,O (Aldrich) and was kept at 27 °C. "H chemical shifts (δ) are expressed in parts/million downfield from internal DSS, but were actually measured by reference to internal acetone (6.2255 in D2O at 27 °C) or free acetate (δ 1.908) in D2O at 27 °C, with an accuracy of 0.002 ppm. "H chemical shifts are expressed in parts/million downfield from internal DSS, with an accuracy of 0.002 ppm.

Two-dimensional ("H,"C) COSY spectroscopy was performed in the absolute-value mode (29) using a spectral width of 2400 Hz. The evolution time (t1) was incremented in steps of 416 μs to obtain 256 time domain points, while the mixing time (t2) was kept at 20 μs. Each measurement consisted of 2 K data points. Two-dimensional ("H,"C) Hartmann-Hahn (HOHAHA) spectroscopy was used for determining the spectral width in the "C domain, incrementing τ1 in steps of 400 μs to obtain 512 f.d., each consisting of the sum of 64 transients acquired in 2 K data points. The spectral width was 1050 Hz. The measurement was repeated in a vacuum desiccator over P2O5 at 27 °C. Quadrature detection in τ1 was accomplished by the time-proportional phase increment (TPPI) method (31).

"C-detected 1′′C-H) shift correlation (HETCOR) experiment was conducted (32), using a spectral width of 6250 Hz in the "H domain. incrementing τ1 in steps of 400 μs to obtain 32 f.d., each consisting of the sum of 1000 transients acquired in 2 K data points. A "H-detected ("H,"C) one-bond shift correlation (HMBC) spectrum was recorded in the absolute-value mode as described by Bax and Subrahmanyan (33) using a "H spectral width of 1250 Hz, incrementing τ1 in steps of 60 μs to obtain 128 f.d., each consisting of the sum of 200 transients acquired in 2 K data points. The "H magnetization of protons attached to "C was suppressed solely by phase-cycling of pulses and receiver. "C decoupling during acquisition (t2) was accomplished using the GARP pulse sequence (34). "H-detected ("H,"C) multiple-bond correlation (HMBC) spectroscopy was performed as described (34) with delays λ2 and λ3 set to 3 τ2, 80 μs, respectively, using a "H spectral width of 1250 Hz, incrementing τ2 in steps of 60 μs to obtain 200 f.d., each consisting of the sum of 2 K data points.
of 400 transients acquired in 2 K data points. The $^1{H}$-detected ($^1{H},^1{C}$) shift correlation experiments (HMQC and HMBC) utilized a 5-mm broad-band probe with reversed geometry. The decoupler transmitter was used for $^1{H}$ pulses (reverse mode) and the sample was not spun (compare Refs. 36 and 37).

RESULTS

Hexasaccharide Purification—By modifying our purification procedure published previously (16), and using three separate HPLC techniques, 16 mg of highly purified hexasaccharide was obtained from the cell wall polysaccharide of S. sanguis H1 for further analysis.

Determination of Glycosyl Composition and Linkage Positions—Composition analysis had shown (16) that the repeating oligosaccharide unit consists of Rha, Gal, and Glc in the molar ratio of 2:3:1. The absolute configurations of the glycosyl residues were determined by preparing the (+)-2-butyl glycosides (28, 37). GLC analysis of the trimethylsilylated butyl glycosides showed that the rhamnosyl residues were in the L configuration, while the other glycosyl residues were all D.

Methylation analysis of the oligosaccharide showed it to consist of terminal Rha, 2-linked Rha, 3-linked Gal, 4-linked Glc, and 3-linked reducing Gal (Gal$^+$), in the ratio of 1:1:2:1:1, by virtue of the mass spectra and retention times of the resulting partially methylated alditol acetates (PMAA). The galactosyl residue at the reducing end was identified by GC/MSD analysis of the PMAA derived from the oligosaccharide which was pre-reduced with NaBD$_4$. This procedure resulted in a 3-O-acetyl-1,2,4,5,6-penta-O-methyl-hexitol (galactitol) with a deuterium atom at C1.

$^{29}$C-F$_{\alpha}$ Plasma Desorption Mass Spectrometry—PDMS of the native repeating oligosaccharide unit verified gas chromatographic evidence (16) that the oligosaccharide was a hexasaccharide. The mass of the molecule was 958.9 (C = 12.01115), which corresponds to C$_{22}$H$_{29}$O$_{20}$ as derived from the PDMS spectrum which showed an (M + Na)$^+$ ion at m/z 982. The peracetylated hexasaccharide showed a m/z value of 1716 for the (M + H)$^+$ ion, indicating the presence of 18 acetylated hydroxyl groups (C$_{22}$H$_{29}$O$_{20}$ = 1715.6). These data are consistent with the hexasaccharide being Hex$_1$[dHex]$_3$. In addition, sequence information became available from the spectrum of the peracetylated hexasaccharide (cf. Ref. 38). Fragmentation occurred in several places within the structure providing acylium ions via cleavage at the protonated acetal linkages, as follows:

![Diagram](image)

The pertinent ions are summarized above the spectrum in Fig. 1. The number of acetyl groups shown in each peak was determined from the mass spectrum of a perdeuterioacetylated sample and confirms the ion structures shown. From the fragment at m/z 503, it was deduced that the 2 Rha (dHex) residues were attached to each other, at the non-reducing end of the hexasaccharide. The peak at m/z 1368 expressing the cleavage of the reducing end hexose from the hexasaccharide acetate is surprisingly weak. In its place is a rather strong peak at m/z 620 that must result from cleavage of a fragment containing the last two hexoses, in the "opposite" direction, as follows:

![Diagram](image)

Conceivably, this cleavage results from anchimeric assistance from the trans-oriented 3-acetate of the penultimate glucose (see below) moiety. The same stereochemical consequences would also hold for a penultimate galactose linked at the 3-position which would be expected to undergo cleavage assisted by its trans-2-acetate. The presence of a hexose (galactose) residue at the reducing end was confirmed by comparing the spectrum of the acetylated hexasaccharide (Fig. 1) to the spectrum of the pre-reduced (NaBH$_4$), acetylated hexasaccharide (not shown). Signals at m/z 331 and 620 were no longer

![Graph](image)
present, whereas signals at m/z 375 and 664 appeared in the latter spectrum. Signals at m/z 273, 503, 792, and 1080 were prominent in both spectra. The combined results from chemical and mass spectrometric analyses indicate that the structure of the hexasaccharide is L-Rhap(1–2)L-Rhap–D-Hexp–D-Hexp–D-Hexp(1–S)D-Gal in which two D-Hex are galactosyl residues and the third is a glucosyl residue.

**Completion of Primary Structure Determination by 'H and 13C NMR Spectroscopy**—The 'H NMR spectrum of the hexasaccharide (Fig. 2A) shows seven anomeric resonances. Based on the chemical shifts and coupling constants, four of these resonances, δ 5.18 (J1 = 3.7 Hz), δ 5.10 (J1 = 1.8), δ 5.06 (J2 = 3.0), and δ 4.88 (J1 = 1.9) were tentatively assigned to H1 of α-linked glycosyl residues. The remaining three resonances, δ 4.6, δ 4.53 (J1 = 7.9) and δ 4.42 (J1 = 7.9), were assigned to H1 of β-linked glycosyl residues. The relatively low intensities of the resonances at δ 5.10 and δ 4.38 (relative areas of 0.3 and 0.6, respectively, compared to 1.0 for all the other anomeric resonances) suggest that they were due to the H1 atoms of the α- and β-anomers of the reducing galactosyl residue, respectively. The signal at δ 4.6 which appears as a set of three peaks consists actually of two doublets, δ 4.61 (J1 = 8.6) and δ 4.60 (J1 = 8.6). The two doublets (intensity ratio 1:2) were assigned to the H1 of the hexosyl residue that is linked to the reducing galactosyl residue and were attributed to the α/β-anomerization of this reducing galactosyl residue. This assignment is based on the HMOC and HMBC experiments which are discussed below. The doublet at δ 4.61 occurs when this hexosyl residue is linked to the α-anomer and the other doublet, at δ 4.60, occurs when it is linked to the β-anomer of the reducing galactosyl residue. The resonances at δ 5.10 and δ 4.88 can be assigned to the 2 rhamnosyl residues by virtue of their small J12 coupling constants. That these rhamnosyl residues were involved in α-glycosyl linkages was confirmed by recording a 'H-coupled 13C spectrum of the hexasaccharide (see below). Henceforth, we will use the anomeric proton assignments to distinguish between the seven 'H NMR spin systems, as follows: the resonances at δ 5.18 and δ 4.53 being assigned to the H1 of the α- and β-anomer of the reducing galactosyl residue, these were denoted α-Gal1,4 and β-Gal1,4; the 2 rhamnosyl residues were denoted α-Rhap1,6 and α-Rhap1,6. The doublet at δ 4.42 and the two doublets at δ 4.61 and δ 4.60 were assigned to two β-linked hexosyl residues, β-Hex1,4 and β-Hex1,4, respectively, while...
the remaining resonance at δ 5.06 is assigned to an α-linked hexosyl residue, α-Hex₆₆₆₆.

Further ¹H assignments for each glycosyl residue were determined by the combination of two-dimensional (¹H,¹H) correlation spectroscopy (COSY), and homonuclear Hartmann-Hahn (HOHAHA) spectroscopy (see Fig. 3). The results are explained below and are compiled in Table I.

With regard to the rhamnosyl residues, the COSY experiment showed that α-Rha₆₆⁰, H1 is coupled to H2 at δ 3.97 and that α-Rha₆₆₆₆, H1 is coupled to H2 at δ 5.96. Coupling was also observed between the H6 methyl protons at δ 1.20 and H5 at δ 3.71, and between H6 methyl protons at δ 1.18 and H5 at δ 3.62. This information, together with the HOHAHA results, allowed the complete assignment of the 'H subspectra of both rhamnosyl residues. In the case of α-Rha₆₆⁰, the HOHAHA subspectrum across the H1 resonance at δ 5.10 (Fig. 3) showed, in addition to the H2 multiplet of narrow width (<9 Hz) at δ 3.97, a doublet of doublets at δ 3.87, a multiplet at δ 3.71, and a doublet of doublets at δ 3.40. The narrow width of the H2 doublet of doublets (δ 3.97) is due to the small coupling constants resulting from the equatorial-equatorial relationship of H2 to H1 and its equatorial-axial relationship to H3. The doublet of doublets at δ 3.89 can be assigned to H3 (Jₓ = 5 Hz, Jₜₜ = 10 Hz). The doublet of doublets at δ 3.40 appears as a triplet and can be assigned to H4 (Jₓ = Jₜₜ = 10 Hz). The relatively large coupling constants reflect the diaxial relationship of H4 with both H3 and H5. The multiplet at δ 3.71 is assigned to H5 in accordance with the COSY experiment (see above). Since the COSY experiment also showed that H5 at δ 3.71 is coupled to the H6 protons at δ 1.20, the 'H assignment of α-Rha₆₆₆₆, was completed. The complete 'H assignment of α-Rha₆₆₆₆, was made in an analogous manner.

With respect to the reducing galactosyl residue, the α-Gal₆₆, H2 signal was assigned from the COSY experiment at δ 3.88 due to its cross peak to H1 at δ 5.18. The HOHAHA subspectrum across H1 at δ 5.18 (Fig. 3) shows multiplets at δ 3.88, 3.86, and 4.15. The multiplet at δ 4.15 is assigned to H4. Its relatively downfield position and narrow peak width (<7 Hz) is typical for an equatorial Gal H4 proton which has an equatorial-axial relationship with both H3 and H5. Thus, the remaining multiplet at δ 3.86 is assigned to H3. The subspectrum through H1 does not allow assignment of H5 from α-Gal₆₆, because of the inefficiency of magnetization transfer from H4 to H5 (Jₓ < 1.5 Hz) (36). However, the subspectrum through δ 4.15 revealed an additional multiplet.
that the chemical shifts of the H2 and the resonant H2 and 3.90, downfield position and narrow peak width are characteristic to the chemical shifts for J, constants, obtained from the one-dimensional 'H spectrum 4.09. The multiplet at 6 3.90 has a total width of 8 Hz. This narrow peak width, its chemical shift, together with the and from the set of vicinal coupling constants of its protons, as was not possible to make the

The remaining multiplet at 6 4.42 shows additional multiplets at 6 3.58, 3.62 and 3.50. Typically, the set of relatively large vicinal coupling constants allows magnetization transfer in the glycosyl residue from H1 all the way to H6 and H6' during the mixing period of the HOHAHA experiment. The multiplet at 6 3.58 is particularly broad (40 Hz) and is also the most intense. The multiplets at 6 3.85 and 3.72 appear to be two doublets of doublets and their chemical shifts and coupling constants (J2,cos = −12 Hz) are consistent with the H6 protons of a δ-glucosyl residue. The broad, intense multiplet at 6 3.58 is due to two co-resonant protons, namely H3 and H4. The remaining multiplet at 3.50 is assigned to H5. This assignment is also confirmed by the COSY experiment in which the H6 protons were coupled to the H5 proton at 6 3.50.

Once the 'H spectrum of the hexasaccharide had been assigned, the 13C spectrum was assigned both by a 'H-detected, as well as by a 13C-detected one-bond ('H, 13C) shift correlation experiment (HMBC and HETCOR, respectively). Both spectra showed one-to-one correlations between carbons and the protons that are (are) directly attached to it; the HMBC spectrum has relatively high resolution in the 'H dimension, while the HETCOR spectrum shows better resolution in the 13C domain. The one-dimensional 13C spectrum of the hexasaccharide is shown in Fig. 2B. The derived 13C assignments for the hexasaccharide are included in Table I. In instances where two or more protons had the same chemical shift, it was not possible to make the 13C assignment directly from the J, correlation spectra. For example, the H3s of 3-1Gal- and 3-4Gal- both resonate at δ 3.70. The resonances for the C3s of these 2 galactosyl residues had, however, since the C-H cross-peaks of the reducing galactosyl residue were consistently of lower intensity than the other cross-peaks. Thus, the cross-peak at δ 83.8/δ 3.70 (which was of lower intensity), and that at δ 78.4/δ 3.70 were assigned to the C3s of β-4Gal- and β-Gal- respectively. The C4s of these 2 residues were similarly assigned, based on relative intensity of cross-peaks, since their H4s both resonate at δ 4.09. The H3 and H4 of β-Glc- and the H2 of β-Gal- all resonate at δ 3.58. Connectivities to 13C signals at δ 79.4, δ 75.4 and δ 70.6 are observed. The signal at δ 79.4 was assigned to C4 of β-Glc since methylation data showed that the glucosyl residue is linked at position 4 (see above) and, therefore, this carbon would be shifted further downfield. The literature (40) reports that the C3 resonance of a β-glucosyl residue is consistently found downfield from the C2 of a β-galactosyl residue and, thus, the peaks at δ 75.4 and δ 70.6 were assigned to the C3 of Glc, and C2 of β-Gal-, respectively. Both the H2 and H3 of α-Gal- are resonant at δ 3.90. Since methylation data show that all the galactosyl residues are linked at position 3 the resonance at δ 77.6 was assigned to C3 and at δ 69.0 was assigned to C2. Finally, the H5s of β-4Gal- and α-Rha- both resonate at δ 3.62. The literature (40) shows that

| Table I | δH and 13C NMR chemical shifts for the constituent residues of the repeating hexa- galactosyl residue from the cell wall polysaccharide of S. sanguis H1 |
| Residue | H1 | H2 | H3 | H4 | H5 | H6 |
| α-Gal- | 5.18 | 3.88 | 3.86 | 4.15 | 4.00 | 3.58-3.65 |
| (a) | 93.1 | 69.0 | 80.8 | 70.1 | 72.2 | 62.0 |
| β-Gal- | 4.53 | 3.54 | 3.70 | 4.09 | 3.62 | 3.58-3.65 |
| (a) | 97.8 | 83.8 | 69.5 | 75.9 | 62.0 |
| α-Glc- | 4.60 | 4.61 | 3.33 | 3.58 | 3.50 | 3.72, 3.85 |
| (b) | 104.8 | 74.1 | 75.4 | 79.4 | 75.7 | 61.1 |
| β-Gal- | 4.42 | 3.68 | 3.49 | 3.63 | 3.58-3.65 |
| (c) | 104.0 | 76.6 | 78.4 | 66.0 | 76.2 | 61.9 |
| α-Gal- | 5.06 | 3.90 | 3.90 | 3.99 | 4.11 | 3.71, 3.62 |
| (d) | 96.5 | 69.0 | 77.6 | 70.5 | 72.0 | 62.1 |
| α-Rha- | 5.10 | 3.97 | 3.85 | 3.49 | 3.71 | 1.20 |
| (e) | 101.8 | 79.1 | 71.5 | 75.2 | 70.4 | 17.5 |
| α-Rha- | 5.98 | 3.96 | 3.96 | 3.34 | 3.62 | 1.18 |
| (f) | 100.3 | 71.1 | 71.2 | 73.2 | 70.4 | 17.5 |

Reserves are denoted by capital letters A-F, indicating their sequence in the hexasaccharide (compare to Fig. 2); the subscripts indicate the chemical shifts of their anomeric protons.

These 'H resonances could not be assigned from the HOHAHA subspectrum (see Fig. 3), their assignment is based on the C-H correlation spectra.

The assignments of these carbon resonances may be interchanged.
Fig. 4. Heteronuclear multiple-bond correlation (HMBC) spectrum of the hexa- saccaride isolated from the cell wall polysaccharide of S. sanguis H1. The selected region shows the long-range $^1H-^1C$ connectivities of the anomeric protons. The structure of the compound and its one-dimensional $^1H$ spectrum are shown on top of the contour map; the $^1C$ projection is shown alongside the vertical axis. Cross-peaks marked by capital letters indicate three-bond couplings ($^3J_{CH}$) across the glycosidic linkages between pertinent residues, those marked by small letters refer to $^2J_{CH}$ and $^3J_{CH}$ couplings within the glycosyl rings. The HMBC spectrum was obtained at 500 MHz, on 14 mg of the oligosaccharide in D.O. 27°C; pD 6; total measuring time: 40 h. Data matrix: 200 × 2048; 409 scans; τ value: squared sine-bell multiplication applied in the $^1H$ dimension, Gaussian line broadening in the $^1C$ dimension, prior to Fourier transformation.

### Table II

Multiple-bond connectivities between the anomeric protons of the constituent glycosyl residue and carbon atoms within the glycosyl residues and across the glycosidic bonds, as observed for the repeating hexa- saccharide unit of S. sanguis H1 cell wall polysaccharide.

<table>
<thead>
<tr>
<th>Cross-peak</th>
<th>$^1H$ Connectivities between</th>
<th>$^1H$ of</th>
<th>Carbon</th>
<th>Residue</th>
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<tr>
<td>ω1-3</td>
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<td>C3</td>
<td>α-Gal,</td>
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<tr>
<td>ω1-2</td>
<td>$^1H$ Gal, β</td>
<td>C2</td>
<td>β-Gal,</td>
<td></td>
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<tr>
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<td>$^1H$ Gal, β</td>
<td>C1</td>
<td>β-Gal,</td>
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<tr>
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<td>C5</td>
<td>α-Gal,</td>
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<tr>
<td>B1-3</td>
<td>$^1H$ Glc</td>
<td>C3</td>
<td>Glc,</td>
<td></td>
</tr>
</tbody>
</table>

Connectivities observed in the HMBC spectrum of the hexa- saccharide (see Figure 4). Bold capital letters indicate $^3J_{CH}$ couplings across the glycosidic bonds; bold small letters indicate $^2J_{CH}$ and $^3J_{CH}$ couplings within a glycosyl ring.

The C5 of a β-galactosyl residue is consistently found downfield from that of an α-rhamnose residue and, therefore, the resonances at δ 75.9 and δ 70.4 are assigned to β-1-Gal,α and α-Rha,α, respectively. Both the proton and carbon assignments for H6 and C6 of α-1-Gal,α, β-1-Gal,β, and β-Gal,β were estimated from the HMQC and HETCOR spectra since these H6 assignments could not be made from the HOHAHA experiment.

The glycosidic linkages of the rhamnose residues were confirmed to have the α-configuration, by recording a $^1H$-coupled $^13C$ spectrum of the hexa- saccharide. From this spectrum, the $J_{CH}$ values for the α-Rha,β, and α-Rha,α C1s (δ 101.8 and 103.3, respectively) were determined to be 171.7 and 170.2 Hz, respectively (40). The ring sizes of all glycosidically linked residues were confirmed to be pyranoses; notably, the absence of anomeric $^13C$-signals around δ 110 ppm (Fig. 2B) points to the linked Gal residues being in the pyranose ring structure (see below).

Once the $^1H$ and $^13C$ spectra had been virtually completely assigned, the sequence of the glycosyl residues in the hexa- saccharide was determined by a heteronuclear multiple bond correlation (HMBC) experiment. In this experiment, inter-glycosidic couplings ($^4J_{CH}$) of the anomeric protons across the glycosyl oxygen to the linked carbon were observed. In addition, intra-ring couplings of the anomeric proton to C3 ($^3J_{CH}$) and in some cases to C2 ($^2J_{CH}$) and C5 ($^4J_{CH}$) were observed. Fig. 4 shows the portion of the HMBC spectrum used for sequencing, and Table II lists the intra- and inter-ring long-range C-H connectivities of the anomeric protons. The se-
The sequence and linkage positions, as determined by HMQC NMR spectroscopy, are in full agreement with the PDMS and methylation data described above.

DISCUSSION

The complete primary structure of the hexasaccharide repeating unit from S. sanguis H1 cell wall polysaccharide has been elucidated by a combination of two-dimensional homonuclear (COSY and HOHAHA) and heteronuclear (HMQC and HMQC) NMR experiments, in conjunction with PDMS and chemical analyses. Given that the oligosaccharide consists of Rha, Gal, and Glc residues in the ratio of 2:3:1, methylation analysis of the deuterio-reduced hexasaccharide showed that a Gal residue is at the reducing end, and PDMS revealed the compound to be a linear hexasaccharide containing the L-Rhap-(1→2)-L-Rhap-(1→3)-α-D-Galp-NAc-(1→6)-D-Galf-(1→6)-D-Gal.

The three S. sanguis strains, H1, 34, and J22 coaggregate with the same actinomyces partners (primary colonizers), but appear to utilize adhesin-to-polysaccharide receptor mechanisms in different ways (47). To establish cell-to-cell contact and maintain the aggregated state in these streptococcal-actinomyces interactions, H1 utilizes an adhesin-like protein, strain 34 utilizes a polysaccharide structure, and J22 utilizes both of these strategies. This diversity in usage of surface structures may allow these strains to be successful, each within its own niche.

Evidence for involvement of the H1 polysaccharide in coaggregation to secondary colonizers came later (48). The H1 to C. ochracea ATCC 33596 interaction was abolished by heating the capnocytophaga but not the streptococcal partner (48), and the interaction was found to be Rha-inhibitable (49). In another study, the H1 polysaccharide was shown to be accessible for cell-to-cell interactions with a second partner while concurrently interacting with actinomyces in experimental dental plaque formation (50). These results suggest that H1 could be simultaneously bound to actinomyces (via H1 adhesin-like interactions) and to capnocytophaga (with the H1 polysaccharide serving as a bridge) in in vitro human dental plaque.

The proposed adhesin binding site on the S. sanguis 34 hexasaccharide consists of the two reducing end sugars, GalNAc(1→3)Gal (51). The most effective saccharide inhibitor of the coaggregation between S. sanguis H1 and C. ochracea ATCC 33596 is Rha (49, 16). While 2 Rha residues are present within the S. sanguis H1 hexasaccharide, the actual site for C. ochracea ATCC 33596 adhesion binding is unknown. Further studies as well as examinations into the identity of the adhesin on C. ochracea ATCC 33596 are ongoing (49; Footnote 2). In addition, the structure of the intact S. sanguis H1 polysaccharide is presently being examined. It is apparent through structural studies such as these that the molecular
mechanisms of coaggregation interactions can be determined. Through analysis of polysaccharide structure, analysis of polysaccharide presentation to its partner cells, and through determination of the adhesion binding fine specificity, differences in coaggregation partner specificities can be explained which may allow many complex interactions found in the human oral ecosystem to be better understood.

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REFERENCES
47. Cisar, J. O., Kolenbrander, P. E., and McIntire, F. C. (1979) "Infect. Immun. 21, 73-95