Structural Elucidation of Two Capsular Polysaccharides from One Strain of *Bacteroides fragilis* Using High-Resolution NMR Spectroscopy†

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ABSTRACT: The capsule of *Bacteroides fragilis* is unusual in that it consists of two distinct capsular polysaccharides. Using a combination of high-resolution NMR spectroscopy, theoretical calculations, and as few chemical procedures as required, the structure of both polysaccharide antigens (polysaccharides A and B) was elucidated. Using the above procedures, it was possible to obtain the complete structures using minimal quantities of polysaccharides A and B (8 and 5 mg, respectively). Only small amounts of each subjected to chemical analysis were not recoverable. Polysaccharide A is composed of the following repeating unit: [→3)α-D-AAT*p*(1→4)[β-D-Gal*f*(1→3)]α-D-Gal*p*NAc(1→3)β-D-Gal*p*(1→], where AAT is 2-acetamido-4-amino-2,4,6-trideoxygalactose. A pyruvate substituent having the *R* configuration spans O-4 and O-6 of the β-D-galactopyranosyl residue. Polysaccharide B is composed of the following repeating unit: [→4)α-L-Qui*p*NAc(1→3)β-D-Qui*p*NAc(1→4)[α-L-Fuc*p*(1→2)β-D-Gal*p*A(1→3)β-D-Glc*p*NAc(1→3)]α-D-Gal*p*(1→]. A 2-aminoethylphosphonate substituent is situated on O-4 of the *N*-acetyl-β-D-glucopyranosyl residue.

Bacteroides fragilis is the most common obligately anaerobic bacterial species isolated from serious human infections (Polk & Kasper, 1977), and studies conducted in animals have demonstrated that its capsule, identified as a high molecular weight polysaccharide (Kasper, 1976), is an important virulence factor (Onderdonk et al., 1977). The capsule has been found to promote the formation of intraabdominal abscesses even in the absence of viable bacteria (Onderdonk et al., 1977), and, in addition, when injected in animals, the polysaccharide is also able to induce in them a T-cell dependent response that provides protection against subsequent challenge with live bacteria (Shapiro et al., 1982). Therefore a precise definition of the *B. fragilis* polysaccharide is essential to understand the structural basis of these important biological properties.

Chemical analysis of the *B. fragilis* polysaccharide indicated that it was complex, being composed of at least six different sugars (Kasper et al., 1983). However, more recent studies (Pantosti et al., 1991) have demonstrated that the complexity of the capsule is not due to the multiplicity of sugars alone but more importantly is due to the fact that the capsule consists of two distinct polysaccharides. A partial separation of the two polysaccharides, designated polysaccharides A and B, was effected by ion-exchange chromatography, although this separation did require that the capsule be subjected to mild acid treatment. The individuality of each polysaccharide was demonstrated by the fact that they both had different sugar analyses and immunoelectrophoretic and antigenic properties.

More recently, a more satisfactory separation of polysaccharides A and B was achieved by subjecting the capsule directly to isoelectric focusing (Tzianabos et al., 1991), thus avoiding the acid treatment step. A comparison by ^{1}H NMR spectroscopy of polysaccharides A and B obtained from this latter procedure with the equivalent preparations obtained from the acid-treated capsule indicated that while the acid treatment did not affect polysaccharide A, it did result in some degradation of polysaccharide B (Tzianabos et al., 1991). We now report the structural elucidation of polysaccharide A and *B. fragilis*.

Experimental Procedures

Growth of the Organism and Isolation of Polysaccharides A and B. B. fragilis NCTC 9343 was obtained from the National Collection of Type Cultures, London, England and was grown anaerobically in broth culture as previously described (Pantosti et al., 1991). The isolation of the capsular material and the subsequent partial separation of its two polysaccharide components (A and B) will be previously described in a separate publication (Tzianabos et al., manuscript in preparation).

Instrumental Methods. ^{13}C, ^{1}H, and ^{31}P NMR spectra were recorded on a Bruker AMX500 or AMX600 spectrometer using a broad-band probe with the ^{1}H coil nearest to the sample. ^{13}C, ^{1}H, and ^{31}P NMR spectra were recorded at 338 and 358 K in 5-mm tubes at concentrations of 1–8 mg of polysaccharide in 0.5 mL of D_2O at neutral pH. Phosphoric acid (25%) was used as an external chemical shift reference for ^{31}P NMR (δ 0.00 ppm). Acetone was used as an internal chemical shift reference for ^{1}H NMR (δ 2.225 ppm) and for ^{13}C NMR (δ 31.07 ppm). All experiments were carried out without sample spinning and with the standard software provided by Bruker. Proton spin simulation with a line width of 2 Hz was performed with the program LAOCN5 available in Dennis Hare's program FTNMR. Coupling constants used in the simulations were taken from the corresponding monomers. Typical parameters for the two-dimensional experiments used in this work were as follows.

COSY (Aue et al., 1976) was recorded in the absolute value mode with a 2500-Hz spectral width. The initial (t_1, t_2) matrix of 512 × 2048 data points was zero-filled to 2K × 2K points to give a digital resolution of 1.2 Hz/point. Unshifted sine-bell filtering in t_1 and t_2 was used, and the spectrum was symmetrized about the diagonal after Fourier transformation.

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Table I: ^{1}H NMR Chemical Shift Data of the *B. fragilis* A Polysaccharide and Appropriate Monosaccharides[a]

sugar residue	chemical shifts								
	H-1	H-2	H-3	H-4	H-5	H-6	H-6	*N*Ac-Me	pyr-Me
(a) →4)α-D-Gal*p*NAc[3→](1→	5.351 [3.0] (5.28)[b]	4.598 (4.19)	4.115 (3.95)	4.212 (4.05)	4.229 (4.13)	3.704 (3.79)	3.766 (3.79)	2.056[c] (2.06)	
(b) β-D-Gal*f*(1→	5.099 [2.9] (5.22)	4.025 (4.02)	4.068 (4.10)	4.005 (4.05)	3.813 (3.81)	3.666 (3.64)	3.690 (3.70)		
(c) →3)α-D-AAT*p*(1→	5.063 [3.5]	4.239	4.444	3.961	4.727	1.340		2.069[c]	
(d) →3)β-D-Gal*p*[(4→pyr), (6→pyr)](1→	4.692 [7.2] (4.58)	3.771 (3.50)	3.805 (3.64)	4.499 (3.94)	3.630 (3.69)	3.955[c] (3.75)	4.077[c] (3.78)		1.477 (1.52)

[a] Measured at 338 K in D_2O with acetone as internal chemical shift reference (δ 2.225 ppm). $^3J_{H,H}$ values are in brackets, and chemical shifts of appropriate monosaccharides are in parentheses. [b] Chemical shifts of monosaccharides are taken from following references: α-D-Gal*p*NAc (Jansson et al., 1989); 4,6-pyruvate (Garegg et al., 1980). No chemical shift data were available for the monosaccharide corresponding to residue c (α-D-AAT*p*). [c] Tentative assignments.

Chemical shifts for overlapping signals in the ^{1}H NMR spectrum were obtained from their cross peaks in a COSY spectrum with 0.5 Hz/point digital resolution.

The HOHAHA experiment (Bax & Davis, 1985) was recorded in the phase-sensitive mode (TPPI) with an MLEV-17 mixing time of 40 or 80 ms and a sweep width of 2500 Hz. The initial (t_1,t_2) matrix of 512 × 2048 data points was zero-filled to give a final matrix of 1K and 2K points with 2.4 and 1.2 Hz/point digital resolution. A π/3-shifted sine-squared bell filter was applied in both dimensions.

A mixing time of 150 ms was used in the 2D NOESY spectrum (Bodenhausen et al., 1984). The spectral parameters were the same as in the HOHAHA experiment described above, except that a π/2-shifted sine-squared bell filter was applied in both dimensions. ^{1}H detection was used for the ^{1}H–^{31}P correlated HMQC recorded in absolute value mode with water suppression and phosphorous decoupling in f_1 using the GARP sequence. The sweep width was 7100 Hz in f_1 (^{31}P) and 4800 Hz in f_2 (^{1}H), and the initial (t_1,t_2) matrix of 128 × 2K data points was zero-filled to 256 × 2K points, which gave a digital resolution of 28 and 2.3 Hz/point, respectively. A π/2-shifted sine-squared bell filter was applied in both dimensions. A ^{1}H–^{13}C HMQC without carbon decoupling in f_1 was performed to determine the $^1J_{C,H}$ coupling constants for the anomeric carbons. The resolution in the f_2 dimension was increased to give a final resolution of 0.6 Hz/point.

The long-range heteronuclear shift correlation experiment, HMBC, was done according to Bax and Summers (1986) in the absolute value mode. The sweep width was 15000 Hz in f_1 (^{13}C) and 2500 Hz in f_2(^{1}H), and the original 256 × 1K data matrix was zero-filled to a final matrix of 512 × 1K points, which gave the same resolution as in the HMQC experiment. The total measuring time was 16 h with 192 scans per t_1 value. Different delays for the evolution of long-range couplings were used (40, 70, and 100 ms). Either Gaussian or π/2-shifted sine-squared bell filters were used for the resolution enhancement.

Deamination of Polysaccharide A. The polysaccharide (2 mg) dissolved in water (100 μL) was treated with 33% (v/v) acetic acid (150 μL) and 5% (w/v) sodium nitrite (150 μL) at room temperature for 40 min and diluted with water (2 mL). After being freeze-dried, the polysaccharide was butanolyzed and analyzed by GC-MS as described below.

Butanolysis and GC-MS. The polysaccharides were first hydrolyzed with 4 M TFA for 1 h at 125 °C, and then the released glycoses were butanolyzed with *R*-(−)-2-butanol (Aldrich) and TFA as the acid donor. Authentic standards of the individual sugars were butanolyzed with (±)-2-butanol. For determination of the D configuration of the 2-acetamido-4-amino-2,4,6-trideoxygalactopyranoside residue, a synthetic sample of L-quinovosamine (2-acetamido-2,6-dideoxy-L-glucopyranose) was used as the standard for its deamination product (i.e., D-quinovosamine). The acetylated butylglycoside derivatives were then analyzed by GC-MS using a Hewlett-Packard 2985 B system and a fused silica OV-17 capillary column (Quadrex Corp.). The identity of each glycose derivative was established by comparison of its GLC retention time and MS with that of the authentic reference samples.

N-Acetylation of the Polysaccharide A. *N*-Acetylation was achieved by treating the polysaccharide with acetic anhydride in 5% (w/v) $NaHCO_3$ (aq).

Determination of the 2-Aminoethylphosphonate. Polysaccharide A was hydrolyzed for 1 h at 120 °C with 4 M TFA. The released glycose residues were analyzed by GC-MC as TMS derivatives. An authentic reference sample of 2-aminoethylphosphonic acid, purchased from Aldrich (order no. 26,867-4), was used as standard for the identification of 2-AEP.

Results and Discussion

Identification of Constituent Sugars and Substituents of Polysaccharide A. Two sugars, D-galactose and 2-acetamido-2-deoxy-D-galactose were identified following sugar analysis and determination of absolute configuration. A complete assignment by two-dimensional methods of all proton and carbon signals in the NMR spectra then led to the identification of all the components of the polysaccharide. The ^{1}H signals were first assigned by using COSY and HOHAHA, the anomeric protons being used as unambiguous starting points in the analyses. Anomeric resonances was labeled a–d in order of chemical shifts. Additional confirmation of the assignments was obtained from a proton spin simulation since the sum of the simulated spectra for each residue was identical to the experimental spectrum (Figure 1). A ^{1}H–^{13}C shift correlated experiment, HMQC, permitted assignment of all carbon atoms that are directly bonded to protons. The ^{1}H and ^{13}C NMR chemical shifts and selected coupling constants for the components in the polysaccharide together with their corresponding monosaccharides are given in Tables I and II.

This analysis showed that the polysaccharide A consists of three hexopyranoses, one hexofuranose, and a substituent identified as a pyruvic acid acetal. Three signals at 49–56 ppm in the carbon spectrum were assigned to nitrogen-bearing carbons. Two singlets in the proton spectrum, each integrating for three protons at 2.1 ppm, together with two signals in the 23 ppm region of the carbon spectrum, suggested that two of the three nitrogens were N-acetylated. The remaining nitrogen was assumed to exist as a free amino group. A doublet (3 H) at 1.3 ppm together with a carbon signal at 17 ppm showed

Table II: ^{13}C NMR Chemical Shift Data of the *B. fragilis* A Polysaccharide and Appropriate Monosaccharides[a]

sugar residue	chemical shifts C1	C2	C3	C4	C5	C6	*N*Ac-Me	*N*AcC=O
(a) →4)α-D-Gal*p*NAc[3→](1→	95.21 [171] (91.95)[b]	49.79 (51.16)	76.24 (68.40)	78.17 (69.56)	73.05 (71.36)	61.34 (62.11)	23.42[c] (22.91)	175.59[c] (175.43)
(b) β-D-Gal*f*(1→	109.96 [170] (102.02)	81.87 (82.36)	77.06 (76.94)	83.23 (83.18)	71.68 (71.69)	63.82 (63.65)		
(c) →3)α-D-AAT*p*(1→	98.85 [171]	49.68	76.05	55.97	64.18	17.05	23.08[c]	175.68[c]
(d) →3)β-D-Gal*p*[(4→pyr), (6→pyr)](1→	105.27 [161] (97.35)	69.76 (72.89)	76.05 (73.74)	68.24 (69.67)	67.22 (75.93)	66.26 (61.84)		
4,6-pyruvate	175.42[c] (174.3)	102.23 (99.8)	26.17 (26.0)					

[a] Measured at 338 K in D_2O with acetone as internal chemical shift reference (δ 31.07 ppm). $^1J_{C,H}$ values are in brackets, and chemical shifts of appropriate monosaccharides are in parentheses. [b] Chemical shifts of monosaccharides taken from the same reference as in Table I. [c] Tentative assignments.

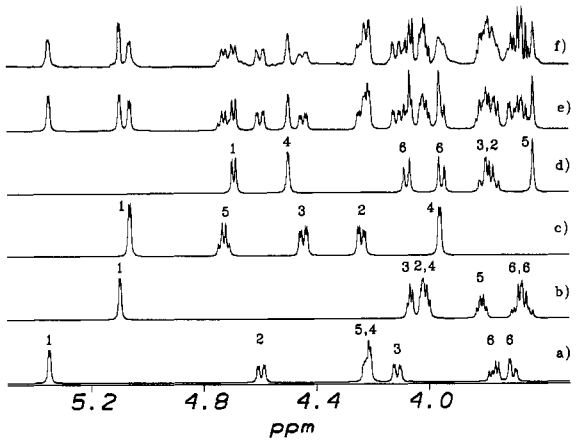


FIGURE 1: Comparison of the simulated and experimental spectra of polysaccharide A. Simulations for each individual spectrum are shown in spectra a–d and their sum in spectrum e. The experimental spectrum is shown in f.

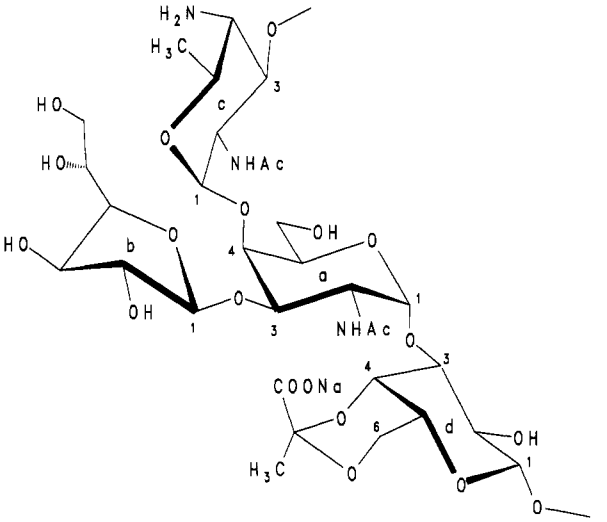


FIGURE 2: Structure of polysaccharide A from *B. fragilis*. The residues are named a–d in order of the ^1H NMR chemical shift of their respective anomeric protons.

that one of the hexoses had a 6-deoxy function.

Residue a (Figure 2) was assigned to 2-acetamido-2-deoxy-α-D-galactopyranose. The coupling constants for the anomeric carbon and proton, $^1J_{C,H}$ of 171 Hz and $J_{1,2}$ of 3.0 Hz, respectively, indicated that this sugar was α-linked, and the $J_{H,H}$ coupling pattern which included small couplings to H_4 showed that this unit had the galactopyranosyl configuration. A chemical shift of 49.8 ppm for the C2 signal was indicative of an acetamido group, and comparison of the chemical shifts of this unit in the polysaccharide with those of its corresponding reducing monomer (Table II) gave further information about linkage positions. A downfield shift for the signals of C3 and C4 (7.8 and 8.6 ppm, respectively) demonstrated that this residue was a branch point in the polysaccharide with linkages to both O-3 and O-4.

Residue b was assigned to a terminal β-D-galactofuranose residue because the chemical shift of the anomeric carbon (109.96 ppm) was indicative of β-furanosides (Table II). Also all proton and carbon resonances, except those from the anomeric center, had chemical shifts that were similar to those of β-D-galactofuranose and only galactose with the D configuration was found in the chemical analysis.

Residue c was assigned to 2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranose (AAT*p*). The coupling constants for the anomeric carbon $^1J_{C,H}$ of 171 Hz and proton $J_{1,2}$ of 3.0 Hz identified this residue as α-linked. Signals at 49.68 and 55.97 ppm for C2 and C4, respectively, indicated that both of these carbons were linked to nitrogens. Since there are only two *N*-acetyl groups in the polysaccharide and one had been previously assigned to residue a (Gal*p*NAc), one of the signals therefore must correspond to a free amino group. In order to determine which one of the two signals was due to the free amino group, the polysaccharide was *N*-acetylated and its proton spectra analyzed by COSY and HOHAHA experiments. An additional signal at 2.1 ppm (3 H singlet) in the spectrum accounted for the new *N*-acetyl group. The downfield shift of the signal for H4 (0.5 ppm) together with upfield shifts for H3 and H5 (0.1 ppm) showed that the free amino group was located at the C4 position. All other protons showed no significant δ changes (<0.05 ppm). Because of the small coupling constant of H4 of ~1 Hz it was possible to assign the galacto configuration to this sugar. It was also deduced from NOE's and chemical shift analysis, which will be discussed later, that this sugar also had the D configuration. It was not possible to determine any linkage position to residue c from chemical shifts since no reference monosaccharide was available, but unless it is terminal, it must be linked at O-3.

Additional evidence for the D configuration of residue c was obtained when the polysaccharide was deaminated with sodium nitrite and butanolyzed to yield the butyl glycoside of quinovosamine (2-acetamido-2,6-dideoxyglucose), the product of inversion of the configuration of residue c at C4. An identical inversion of configuration of AAT at C4 had been demonstrated earlier by Jennings et al. (1980), Kenne et al. (1980), and Lindberg et al. (1980). GC-MS analysis of the butyl glycosides in comparison with an authentic reference sample of quinovosamine confirmed that residue c (AAT*p*) had the D configuration.

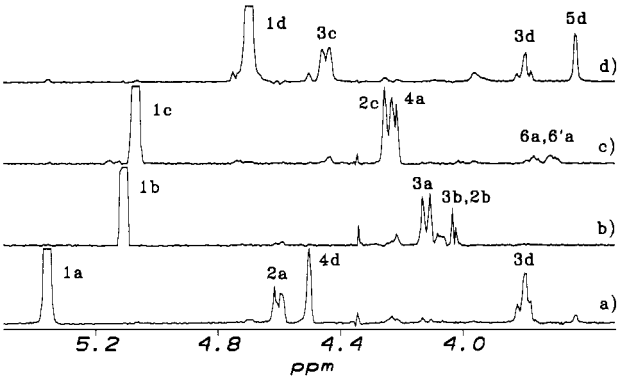


FIGURE 3: Cross sections from a 2D NOE spectrum of polysaccharide A showing the NOE's for the anomeric protons of residues a–d. The mixing time was set to 150 ms in this experiment.

Residue d was assigned to β-D-galactopyranose with a pyruvic acid acetal substituent. The β-linkage was determined from the anomeric coupling constants $^1J_{C,H}$ of 161 Hz and $J_{1,2}$ of 7.2 Hz, and the small coupling to H4 of ~1 Hz gave the *galacto* configuration. Since only D-galactose was found in the chemical analysis, it was possible to conclude that residue d was β-D-galactopyranose. The large differences in chemical shifts compared with those of the corresponding monosaccharide indicated that the pyruvic acid acetal was linked on O-4 and O-6 of this residue (Figure 2). A long-range coupling between the acetal carbon and one of the H6 protons confirmed the location of O-4 and O-6 of the pyruvate. Both H6 protons had small couplings to H5 (~1 Hz) showing that the hydroxymethyl group was locked in a gauche–gauche conformation with both hydroxymethyl protons cis to H5. The chemical shift of the methyl group in the pyruvate was indicative of the stereochemistry (Garegg et al., 1980). In this case the chemical shifts δ_H of 1.48 and δ_C of 26.17 ppm indicated an *R* configuration.

Structure of Polysaccharide A. The structure of polysaccharide A is shown in Figure 2. After unambiguous assignment of all proton and carbon signals, two different NMR methods were used to determine the sequence of the sugar residues.

The nuclear Overhauser effect is widely used for obtaining sequence information because there is generally close proximity between the anomeric proton and the proton attached to the carbon across the glycosidic linkage. NOE's to adjacent protons can also be assistance in the determination of the absolute configuration of an unknown sugar residue. Cross sections for the anomeric protons from the 2D NOE spectrum are shown in Figure 3. Long-range ^1H–^{13}C couplings across the glycosidic linkage give accurate sequence information and may be obtained in the proton-detected HMBC experiment (Figure 4). This method was previously applied to a polysaccharide by Byrd et al. (1987).

Three of the four glycosidic linkages could immediately be established by the use of the HMBC experiment (Figure 2). A long-range coupling from H1-b to C3-a and from C1-b to

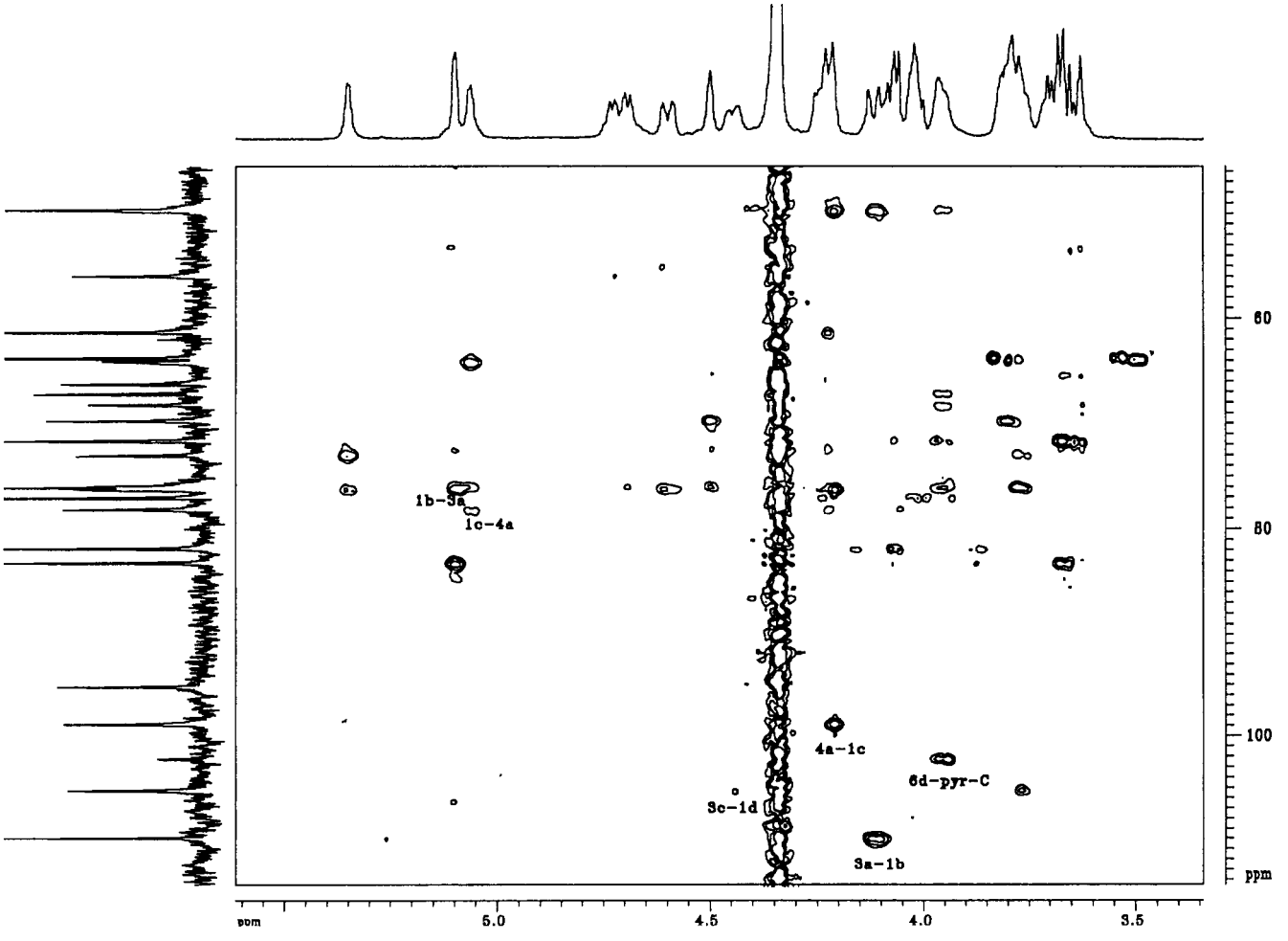


FIGURE 4: Long-range ^1H–^{13}C correlated spectrum (HMBC) of polysaccharide A. Indicated are the observed interresidue connections. The delay for evolution of long-range couplings was set to 70 ms, and total measurement time was 16 h. The cross peak named 6d-pyr-C is due to the coupling between H6 of residue d and the quartenary carbon of the pyruvic acid acetal.

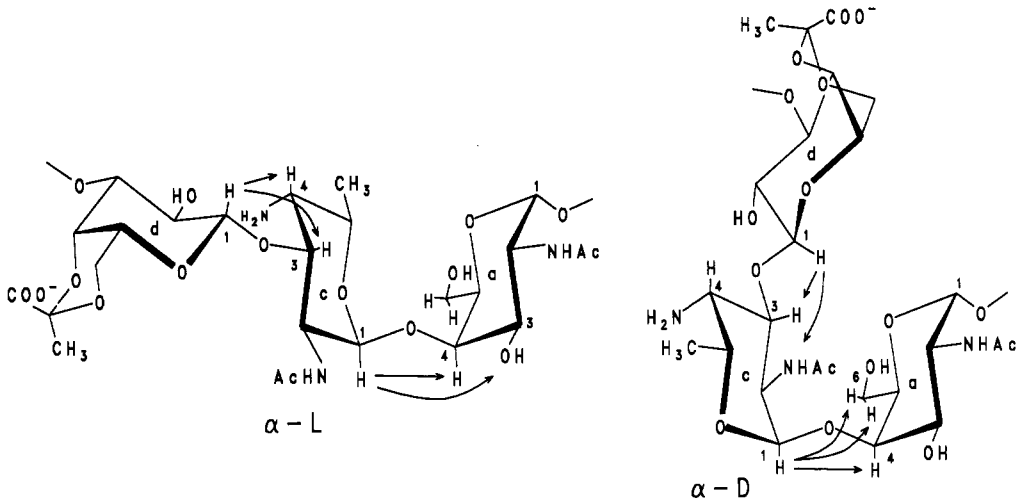


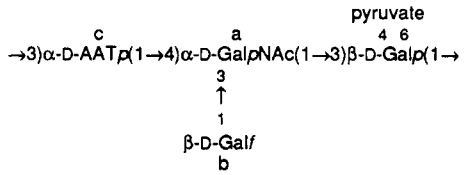
FIGURE 5: Proximities for anomeric protons of residues c and d as obtained from GESA calculations when residue c (α-AAT*p*) has either the L or D configuration.

H3-a showed that residue b (β-D-Gal*f*) was linked to O-3 of residue a (α-D-Gal*p*NAc). The same information was obtained from the 2D NOE spectrum where a cross peak between H1-b and H3-a is observed (Figure 3). Couplings from H1-c to C4-a and C1-c to H4-a indicated that residue c (α-D-AAT*p*) was linked to O-4 of residue a, thus confirming that the GalNAc was a branched point in the polysaccharide. In the 2D NOE spectrum, the expected cross peak between H1-c and H4-a was observed together with cross peaks also to both hydroxymethyl protons of residue c. Finally, a coupling between C1-d and H3-c showed that residue d (β-D-Gal*p*) was linked to O-3 of residue c. The linkage between H1-d and C3-c could not be determined since the signals for C3-c and C3-d had identical chemical shift. However, the expected cross peak between H1-d and H3-c was readily observed in the 2D NOE spectrum (Figure 3). The remaining linkage could not be determined from HMBC, because no interresidue correlations were observed from the anomeric proton or carbon of residue a. Thus, this linkage could only be determined from the 2D NOE spectrum. Cross peaks from H1-a were observed both to H3 and H4 of residue d (β-D-Gal*p*), and it has been shown that the anomeric proton of an α-D-sugar linked to O-3 of a sugar with the D-galacto configuration is close to both H3 and H4 (Lipkind & Kochetkov, 1984). In addition, since both O-4 and O-6 of residue d were occupied by the pyruvic acetal, this leaves O-3 as the only possible linkage site.

Conformational behavior in the vicinity of the glycosidic linkage can be estimated with help of potential energy calculations (Bock, 1983), and this in combination with NMR can be used to determined the absolute configuration of constituent monosaccharide residues in an oligo- or polysaccharide, provided the absolute configuration of the adjacent sugar is known. It was shown by Baumann et al. (1988) that a change in absolute configuration of one of the sugars in a disaccharide is well reflected in the chemical shifts and in the interatomic interactions. If there are unique proton–proton proximities present, they can be detected with the help of NOE experiments, and thus the anomeric configuration can be established.

Because of its sensitivity to acid hydrolysis and the lack of reference compounds, the absolute configuration of residue c (AAT*p*) could not be determined directly by chemical methods. It was known from NMR studies that AAT*p* had the galacto configuration, was substituted at C3 by β-D-galactose, and had an α(1–4) linkage to 2-acetamido-2-deoxy-D-galactose. The interresidue proximities for the two disaccharide elements are shown in Figure 5, where AAT*p* (residue c) has either the L or D configuration; and although the glycosidic linkages between the sugars are flexible, from potential energy calculations (Paulsen et al., 1985), the observed interresidue NOE's can only arise if residue c has the D configuration. In this case, the minimum energy conformers (Figure 5) can be used to explain the experimentally observed NOE's. If AAT*p* had the D configuration, an NOE (Figure 3) between H1-d and H3-c should be observed, but none between H1-d and H4-c since H1-d in this case would be directed toward the *N*-acetyl group. In the alternate L configuration, both sets of the above NOE's would be observed (Figure 5). Additionally, H1-c had NOE's to both H6-a and H6′-a due to the fact that in the D configuration the anomeric proton of residue c is directed toward the hydroxymethyl group of residue a (Figure 5), whereas in the L configuration it would be directed toward O-3 (Figure 5). All the observations are in agreement with those observed in studies on related oligosaccharides (Baumann et al., 1989). The D configuration of AAT*p* was also confirmed by GLC analysis of the butyl glycoside of its quinovosamine derivative. This derivative was obtained by the deamination and subsequent butanolysis of the polysaccharide as previously described.

Hence, the structure of polysaccharide A from *B. fragilis* was found to be



where AAT is 2-acetamido-4-amino-2,4,6-trideoxy-α-D-Gal*p*.

Identification of Constituent Sugars and Substituents of Polysaccharide B. The ^1H and ^{13}C NMR chemical shifts and selected coupling constants for the components in polysaccharide B together with their corresponding monosaccharides are given in Tables III and IV. The residues are labeled a–f according to the order of the chemical shift for the anomeric protons. A close examination of the ^1H and ^{13}C NMR spectra revealed that polysaccharide B consisted of six sugars, and integration of the ^1H spectrum showed that they are in equimolar amounts. This is in agreement with the work of Kasper et al. (1983), who found the following six sugars: L-fucose, D-galactose, D- and L-quinovosamine, D-glucosamine, and galacturonic acid in polysaccharide B. Our chemical analysis confirmed this result and also demonstrated that

Table III: ^{1}H NMR Chemical Shift Data of the *B. fragilis* B Polysaccharide and Appropriate Monosaccharides[a]

sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	*N*Ac-Me
(a) α-L-Fuc*p*(1→	5.200 [3.5] (5.20)[b]	3.779	3.857 (3.77)	3.794 (3.86)	4.378 (3.81)	1.304 (4.20)	 (1.21)	
(b) →4)α-D-Gal*p*[3→](1→	5.063 [nr] (5.26)	3.729 (3.81)	3.882 (3.85)	4.303 (4.00)	4.197 (4.08)	3.662 (3.74)	3.702 (3.74)	
(c) →4)α-L-Qui*p*NAc(1→	5.007 [nr] (5.14)	3.973 (3.88)	3.755 (3.70)	3.335 (3.22)	4.264 (3.91)	1.316 (1.27)		2.049[c] (2.04)
(d) →3)β-D-Qui*p*NAc(1→	4.850 [8.0] (4.68)	3.909 (3.67)	3.602 (3.48)	3.273 (3.22)	3.447 (3.48)	1.332 (1.30)		2.037[c] (2.04)
(e) →2)β-D-Gal*p*A(1→	4.777 [7.5] (4.56)	3.552 (3.51)	3.873 (3.69)	4.253 (4.23)	3.991 (4.03)			
(f) →3)β-D-Glc*p*NAc[4→2-AEP](1→	4.583 [8.1] (4.72)	3.970 (3.65)	4.314 (3.56)	4.158 (3.46)	3.591 (3.46)	3.893 (3.75)	3.934 (3.91)	2.002[c] (2.06)
2-aminoethylphosphonate	2.067, 2.313 (CH_2-P)	3.363, 3.437 (CH_2-N)						

[a] Measured at 358 K in D_2O with acetone as internal chemical shift reference (δ 2.225). $^3J_{H,H}$ values are in brackets, and chemical shifts of appropriate monosaccharides are in parentheses. [b] Chemical shifts of monosaccharides are taken from following references: α-L-Fuc*p*, β-D-Gal*p*A, and β-D-Glc*p*NAc (Jansson et al., 1989). [c] Tentative assignment.

Table IV: ^{13}C NMR Chemical Shift Data of the *B. fragilis* B Polysaccharide and Appropriate Monosaccharides[a]

sugar residue	C1	C2	C3	C4	C5	C6	*N*Ac-Me	*N*Ac-CO
(a) α-L-Fuc*p*(1→	101.03 [174] (93.12)[b]	69.92 (69.09)	70.94 (70.30)	73.60 (72.80)	67.94 (67.10)	17.14 (16.33)		
(b) →4)α-D-Gal*p*[3→](1→	100.80 [173] (93.18)	68.90 (69.35)	81.42 (70.13)	77.12 (70.28)	71.81 (71.30)	62.18 (62.04)		
(c) →4)α-L-Qui*p*NAc(1→	98.97 [171] (92.13)	55.36 (55.76)	71.27 (71.87)	84.67 (77.09)	68.70 (69.01)	18.13 (18.17)	23.99[c] (23.23)	174.81[c]
(d) →3)β-D-Qui*p*NAc(1→	102.39 [163] (96.18)	56.93 (58.37)	81.27 (75.08)	75.58 (76.58)	73.20 (73.39)	18.28 (18.17)	23.99[c] (23.23)	175.20[c]
(e) →2)β-D-Gal*p*A(1→	101.34 [163] (96.89)	78.25 72.62	75.36 73.79	72.01 71.18	77.17 76.44	175.17[c] 175.59		
(f) →3)β-D-Glc*p*NAc[4→2-AEP](1→	104.66 [159] (95.85)	57.21 (57.86)	76.37 (74.81)	73.30 (71.06)	77.06 (76.82)	62.18 (61.85)	23.72[c] (23.10)	175.46[c] (175.49)
2-aminoethylphosphonate	26.95 {CH_2-P} (27.26)	[136Hz $^1J_{C,P}$] (131.8Hz)	37.17 {CH_2-N} (36.74)					

[a] Measured at 358 K in D_2O with acetone as internal chemical shift reference (δ 31.07). $^1J_{CH}$ values are in brackets, and chemical shifts of appropriate monosaccharides are in parentheses. [b] Chemical shifts of monosaccharides are taken from following references: α-L-Fuc*p*, β-D-Gal*p*A, and β-D-Glc*p*NAc (Jansson et al., 1989), and 2-AEP (Glowacki & Topolski, 1989). [c] Tentative assignments.

galacturonic acid had the D configuration. In addition a substituent was also identified as 2-aminoethylphosphonate (2-AEP). This substituent has to our knowledge never been found before in bacterial polysaccharides but has been found in certain glycolipids (Ferguson et al., 1982; Abe et al., 1991).

Following complete assignment of the proton and carbon NMR signals, each spin system could be combined with one of the six sugar residues. The assignment techniques were the same as described previously for polysaccharide A, and a proton spin simulation gave additional confirmation of the assignments. The simulated proton spectra of all components of polysaccharide B are shown in Figure 6, and good agreement was found between the experimental spectrum and the sum of the subspectra. In order to obtain accurate $^1J_{C,H}$ coupling constants, a proton-detected heteronuclear H,C-correlated (HMQC) experiment was perfomed without carbon decoupling and with high resolution (0.6 Hz/point) in the f_2 dimension (^{1}H). From the chemical shifts of the anomeric carbon signals (Table IV), it was evident that all constituent sugars were in the pyranosidic form. Three singlets in the proton spectrum at 2.00–2.05 ppm integrating for a total of nine protons together with three signals in the carbon spectrum at 23.7–24.0 ppm suggested that all three amino sugars were *N*-acetylated.

Residue a was assigned to α-L-fucopyranose, and the coupling constants for the anomeric carbon and proton, $^1J_{C,H}$ of

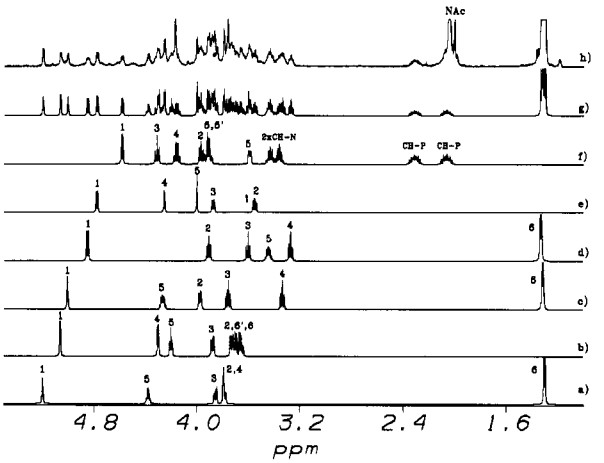


FIGURE 6: Comparison of the simulated and experimental spectra of polysaccharide B. Simulations for each individual spectrum are shown in a–f and their sum in g. The experimental spectrum is shown in h.

174 Hz and $J_{1,2}$ of 3.0 Hz, respectively, indicated that this sugar was α-linked. Small *J* couplings to H4 of ~1 Hz were consistent with the galacto configuration, and a doublet assigned to H6 and integrating for three protons demonstrated that this residue had a 6-deoxy function. Similar ^{13}C NMR

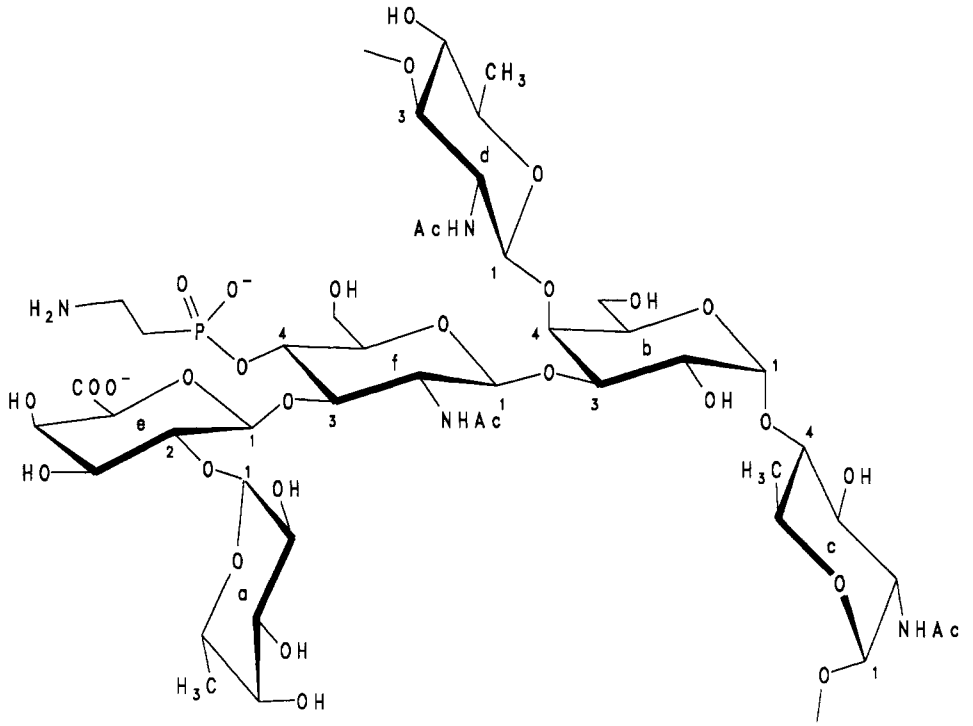


FIGURE 7: Structure of polysaccharide B from *B. fragilis*. The residues are named a–f in order of the ^{1}H NMR chemical shift of their respective anomeric protons.

chemical shifts for this residue in polysaccharide B and those of the corresponding monomer indicated that unit a was a terminal residue.

Residue b was assigned to α-D-galactopyranose. Coupling constants of 173 Hz for $^1J_{C,H}$ and <3 Hz for $J_{1,2}$ together with small couplings to H4 identified this residue as α-linked with the galacto configuration. The signals for C3 and C4 were both shifted downfield 11.3 and 6.8 ppm, respectively, compared with the values in the corresponding monomer (Table IV) suggesting that residue b was a branch point with linkages at O-3 and O-4.

Residue c was assigned to 2-acetamido-2,6-dideoxy-α-L-glycopyranose (quinovosamine). The coupling constants $J_{1,2}$ of <3 Hz and $^1J_{C,H}$ of 171 Hz indicated that it had the α configuration and a chemical shift for the C2 signal at 55.4 ppm demonstrated that C2 was linked to a nitrogen atom. The J coupling pattern with large coupling constants around the ring (~10 Hz) was consistent with the gluco configuration. Examination of the chemical shift differences for the carbon signals of residue c as compared to values in the corresponding monomer indicated that C4 was shifted 7.6 ppm downfield, thus suggesting that residue c was substituted at O-4. At this stage, it was not possible to determine whether this residue had the D or L configuration since chemical analysis showed that polysaccharide B contained two quinovosamine residues, each having different absolute configurations. However, with the help of potential energy calculations using the GESA program (Paulsen et al., 1985) in addition to NOE experiments, they could be distinguished, and this will be discussed later in detail.

Residue d was assigned to the other 2-acetamido-2,6-dideoxy-β-D-glucopyranose (quinovosamine) residue. The large coupling constant for the anomeric proton, $J_{1,2}$ of 8 Hz together with $^1J_{C,H}$ of 163 Hz, demonstrated that residue d had the β configuration. Signals at 56.93 and 18.28 ppm for C2 and C6, respectively, indicated the presence of a nitrogen atom linked to C2 and a deoxy function at C6. A J coupling pattern similar to that of residue c indicated that residue d had the

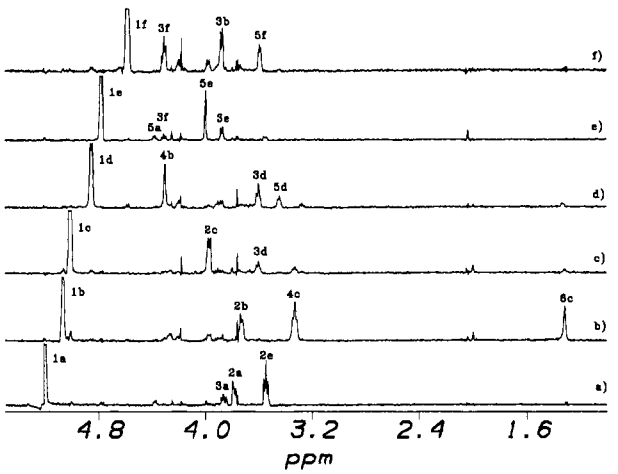


FIGURE 8: Cross sections from a 2D NOE spectrum of polysaccharide B showing the NOE's for the anomeric protons of residues a–f. The mixing time was set to 150 ms in this experiment.

gluco configuration. The 6.2 ppm downfield shift for the C3 signal suggested that residue d was linked at O-3. As in the case of the other quinovosamine (residue c) the absolute configuration of residue c could not be determined at this point.

Residue e was assigned to β-D-galactopyranuronic acid. The β configuration was established from the coupling constants, $^3J_{H1,H2}$ of 7.5 Hz and $^1J_{C1,H1}$ of 163 Hz. This was the only sugar with a spin system consisting of five protons, and the small couplings to H4 made it possible to assign this residue to galacturonic acid. The downfield shift of the C2 signal (5.6 ppm) suggested that this sugar was linked at O-2.

Residue f was assigned to 2-acetamido-2-deoxy-β-D-glucopyranose. Coupling constants of 8.1 and 159 Hz for the anomeric proton and carbon, respectively, indicated the β configuration. The chemical shift of 57.21 ppm for the C2 signal demonstrated the amino function, and the J coupling pattern for the protons with only large (~10 Hz) coupling constants enabled the assignment of the gluco configuration

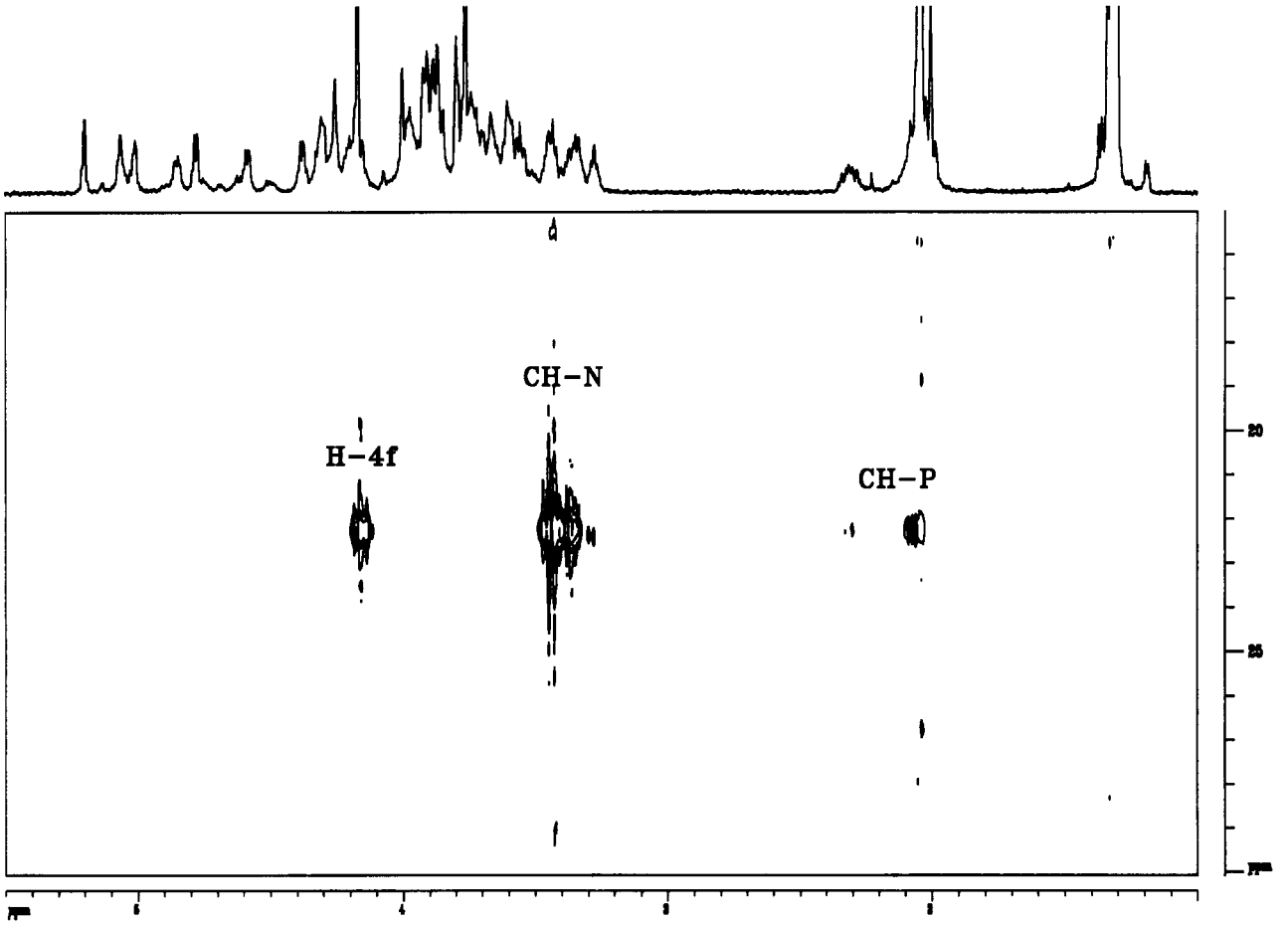


FIGURE 9: ^{1}H–^{31}P correlated spectrum (HMQC) of polysaccharide B. Indicated are the protons which are coupled to the phosphorus atom in 2-AEP confirming that 2-AEP is linked to position 4 of residue f. The experiment was optimized for proton phosphorus couplings of ~10 Hz, explaining the low intensity of the CH_2–P cross peaks ($^{2}J_{H,P}$ ~20 Hz).

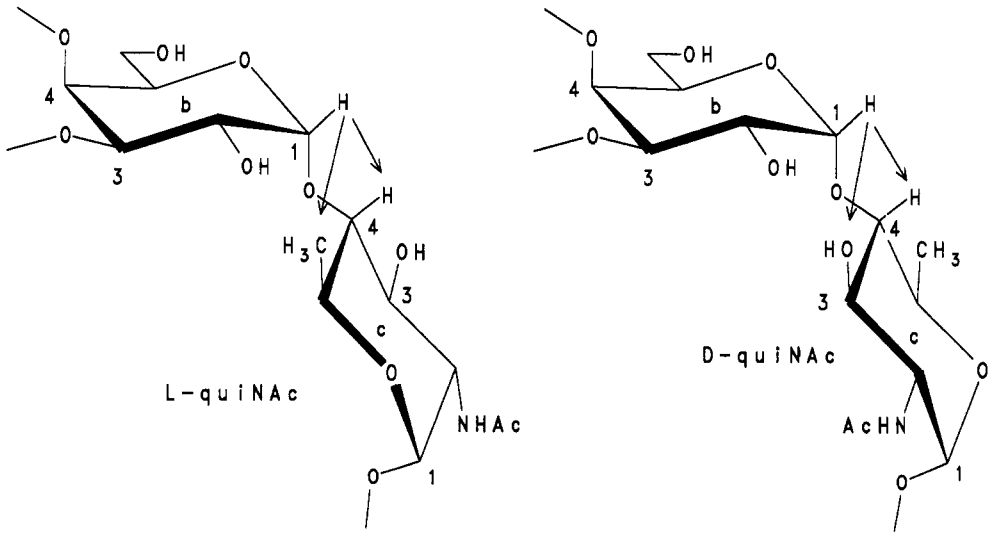


FIGURE 10: Proximities for the anomeric proton of residue b (α-D-Gal*p*) was obtained from GESA calculations when residue c (α-Qui*p*NAc) has either the L or D configuration.

to this residue. Both the C3 and C4 signals were shifted downfield by 1.6 and 2.2 ppm, respectively, and H3 and H4 were shifted downfield by 0.75 and 0.70 ppm, suggesting that O-3 and O-4 were substituted.

The substituent was assigned to 2-aminoethylphosphonate (2-AEP). It had a characteristic ^{13}C NMR spectrum with a 136 Hz coupling constant for the signal at 26.95 ppm due to the one-bond ^{13}C–^{31}P coupling. ^{31}P NMR showed one signal at 22.3 ppm which was very broad when the experiment was performed without proton decoupling but became a sharp peak when proton decoupling was employed. This was due to the fact that five different protons had couplings to the phosphorus atom. The identity of 2-AEP was also determined by GC-MS of its TMS derivative using an authentic reference which had identical retention time and mass spectrum.

Structure of the Polysaccharide B. After determination of all the residues and the substituent, the structure of polysaccharide B, shown in Figure 7, was established with the help

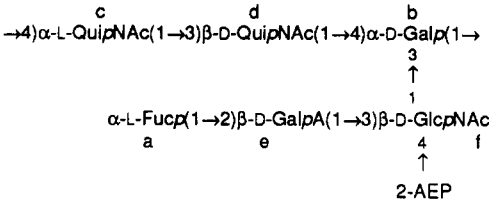
of two different NMR methods. 2D NOE was used to determine the sequence of the sugar residues. Interresidue NOE's were found for all linkages in polysaccharide B, and the linkage positions were in agreement with those found in the ^{13}C NMR chemical shift analysis. Cross sections from the 2D NOE spectrum showing the enhancements from the anomeric protons are shown in Figure 8. Long-range ^{1}H–^{13}C couplings which were used to determine the structure of polysaccharide A could not be observed in this case, probably due either to the broader lines exhibited in the spectra of this sample as compared to polysaccharide A or to the small amount of material used. A ^{1}H–^{31}C correlated experiment, HMQC, was used to establish the position of the substituent 2-AEP.

Starting from the terminal residue in the side chain, a cross peak between H1-a and H2-e was observed in the 2D NOE spectrum. This established that residue a (α-L-Fuc*p*) was linked to O-2 of residue e (β-D-Gal*p*A). The cross peak from H1-e to H3-f indicated that residue e was linked to O-3 of residue f (β-D-Glc*pN*Ac). Residue f was linked to O-3 of residue b (α-D-Gal*p*) in the main chain as indicated by the cross peak between H1-f and H3-b. Although the signals for H3-a, -b, and -e seem very close in chemical shift, they could easily be distinguished using a high-field 600-MHz NMR instrument, thus making the assignment unambiguous. The cross peak between H1-b and H4-c indicated that residue b was linked to O-4 of residue c (α-L-Qui*pN*Ac). Residue c was linked to O-3 of residue d (β-D-Qui*pN*Ac) as indicated by the cross peak between H1-c and H3-d, and the cross peak between H1-d and H4-b showed that residue d was linked to O-4 of residue b.

An additional splitting of the H4 resonance of residue f by 9.5 Hz suggested that 2-AEP was linked to O-4 of this residue. A ^{1}H–^{31}P correlated HMQC (Figure 9) was performed in order to determine this connectivity. Apart from cross peaks between the phosphorus atom to all four protons in 2-AEP, a cross peak to H4-f was observed, thus confirming the position of 2-AEP.

It was evident from the chemical analysis that one of the two quinovosamines had the L and the other had the D configuration. However, to assign these configurations to individual residues using chemical methods would be difficult, especially with the small amount of material available. Instead, potential energy calculations using the GESA program in combination with NOE were used as described for polysaccharide A. From the NMR analysis, it was evident that α-D-Gal*p* (residue b) was linked to O-4 of residue c (α-Qui*pN*Ac). Thus, residue c can have either the L or D configuration, and the two possible disaccharide combinations are shown in Figure 10. In the minimum energy conformers (Figure 10), there would be a close proximity between H1-b and H4-c (~2.3 Å) in both cases. However, if residue c had the L configuration, H1-b would also be close to H6-c (2.4 Å), and an NOE between the two protons should be readily observed. If on the other hand, residue c had the D configuration, H-1b would be in close proximity to O-3c, the distance between H6-c and H1-b would be 4.5 Å, and no NOE should be observed. In fact, a large cross peak between H1-b and H6-c was observed in the 2D NOE spectrum, thus distinguishing between these two possibilities, and the L configuration could be assigned to residue c. After this was established, the second quinovosamine (residue d) must therefore have the D configuration, consistent with the identification by chemical analysis of D quinovosamine in polysaccharide B.

Hence, using a combination of available NMR techniques, a few chemical methods, the theoretical calculations, the structure of polysaccharide B from *B. fragilis* was found to be



where 2-AEP is 2-aminoethylphosphonate.

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Registry No. Polysaccharide A repeating unit, 139583-01-0; polysaccharide B repeating unit, 139583-02-1.

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