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Carbohydrate Research 278 (1995) 143–153

CARBOHYDRATE RESEARCH

Structural studies of the extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain 49

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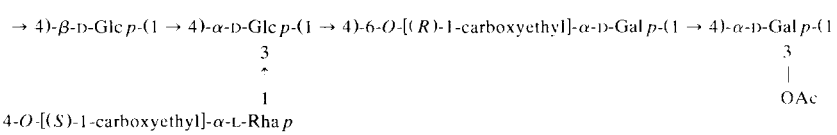
Received 29 March 1995; accepted 18 May 1995

Abstract

The structure of *Butyrivibrio fibrisolvens* strain 49 capsular polysaccharide has been investigated mainly by sugar and methylation analysis, partial chemical degradations, NMR spectroscopy, and mass spectrometry. The results suggest that the polysaccharide is composed of pentasaccharide repeating units having the following structure.



The polysaccharide contains *O*-acetyl groups, one of which is substituted to O-3 of the 4-substituted α-D-Gal*p* residue, while others occur in non-stoichiometric amounts at other locations.

Keywords: *Butyrivibrio fibrisolvens*; Extracellular polysaccharide; Structural elucidation; Bacterial polysaccharide; (1-Carboxyethyl)hexose

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SSDI 0008-6215(95)00234-0

1. Introduction

Butyrivibrio fibrisolvens is a strictly anaerobic bacterial species commonly isolated from the gastrointestinal tract of ruminant animals. Though principally involved in the catabolism of plant-derived polysaccharides, most strains of *B. fibrisolvens* produce significant amounts of extracellular polysaccharides when grown in pure culture [1]. These bacterially produced polymers contain an assortment of unusual monosaccharide constituents such as L-altrose [2], L-iduronic acid [3], several (1-carboxyethyl) sugars [4], and others in a strain-specific manner [1].

Detailed structural characteristics of only the *B. fibrisolvens* strain X6C61 capsular polysaccharide have been reported to date [3]. We continue these studies in order to define more precisely the structures of the repeating units of the major extracellular polysaccharides produced by the many isolates currently classified as strains of *B. fibrisolvens*. Our previous studies with strain 49 have shown the presence of D-glucose and D-galactose, and the less common (1-carboxyethyl)-D-galactose and (1-carboxyethyl)-L-rhamnose [4,5]. We now report further structural studies of this material.

2. Results and discussion

The crude capsular material was prepared as described [6] and then further fractionated by anion-exchange chromatography on DEAE-Sepharose to yield the pure polysaccharide. The ^{1}H and ^{13}C NMR spectra of the polysaccharide were complex and contained, *inter alia*, two signals from *O*-acetyl groups at δ 2.15 and 2.20 together corresponding to five protons. The polysaccharide was *O*-deacetylated and the simplified ^{1}H and ^{13}C NMR spectra (Figs. 1 and 2) together with an HMQC spectrum of the product showed signals from five anomeric protons and carbons, which indicated a pentasaccharide repeating unit. The chemical shifts of these signals and coupling constants, $^{1}J_{C,H}$ and $^{3}J_{H\text{-}1,H\text{-}2}$ (Table 1), indicated that the repeating unit consisted of one β-pyranosyl and four α-pyranosyl residues. The ^{13}C NMR spectrum also contained signals for two carbonyl carbons at δ 181.4 and 181.6, and three methyl carbons at δ 17.9, 19.0, and 19.5 indicating the presence of (1-carboxyethyl)-D-galactose and (1-carboxyethyl)-L-rhamnose, which have been found in previous studies [4,5]. Three signals corresponding to methyl groups were also observed in the ^{1}H NMR spectrum (Table 1), corroborating the presence of the (1-carboxyethyl)hexoses.

Sugar analysis of the *O*-deacetylated polysaccharide indicated the presence of galactose (1 mol) and glucose (2 mol). The D configuration of these was determined by GLC of the trimethylsilylated (+)-2-butyl glycosides [7].

In order to facilitate the sugar and methylation analysis, the polysaccharide was carboxyl-reduced [8] using $NaBH_4$ as the reducing agent. The procedure had to be repeated in order to obtain a high yield in the reduction. The product was hydrolysed, reduced with $NaBH_4$, and acetylated to yield the acetates of glucitol, galactitol, (2-hydroxy-1-methylethyl)hexitol [(1-hydroxyprop-2-yl)hexitol], and 6-deoxy(2-hydroxy-1-methylethyl)hexitol in the relative proportions 1.8:1.0:0.5:1.0, as revealed by

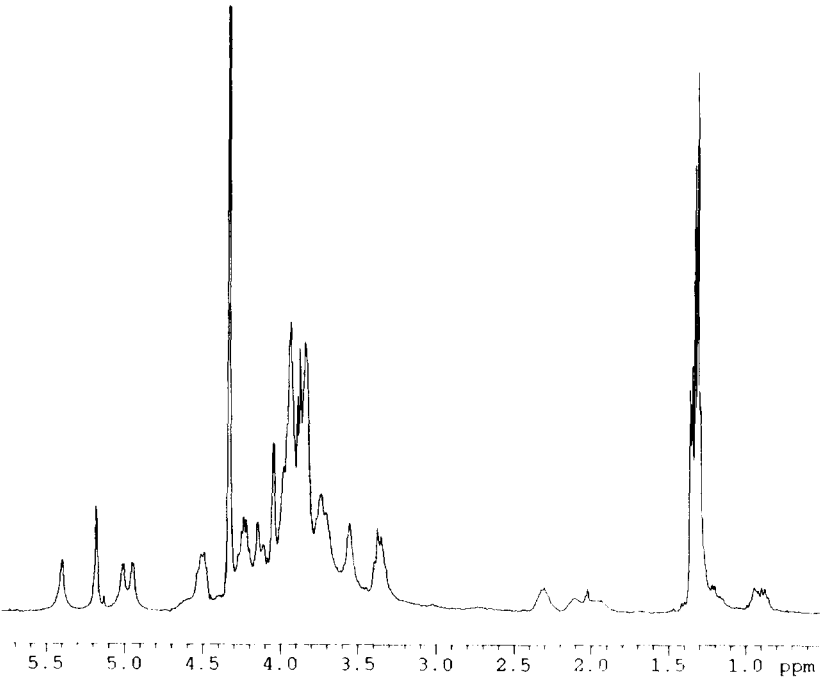

Fig. 1. 400-MHz ^{1}H NMR spectrum of *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain 49.

GLC–MS. The D and L configurations of the (1-carboxyethyl)-D-galactose and the (1-carboxyethyl)-L-rhamnose have earlier been determined [4,5].

Methylation of the *O*-deacetylated polysaccharide was performed using sodium methylsulfinyl anion and CH_3I according to Hakomori [9]. The permethylated polysaccharide was carboxyl-reduced with a solution of lithium triethylborodeuteride (Superdeuteride), according to Bhat et al. [10]. The reduced permethylated polysaccharide was hydrolysed, reduced with $NaBD_4$, and acetylated. The products were analysed by GLC–MS, yielding the deuterium-substituted alditol-*1-d* acetates of 2,3,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-glucose, 6-*O*-(2-hydroxy-1-methylethyl-*2,2-d*$_2$)-2,3-di-*O*-methyl-D-galactose, and 4-*O*-(2-hydroxy-1-methylethyl-*2,2-d*$_2$)-2,3-di-*O*-methyl-L-rhamnose, indicating the repeating unit of the polysaccharide to be composed of 4-substituted D-glucose, D-galactose, and 6-*O*-(1-carboxyethyl)-D-galactose residues, a 3,4-substituted D-glucose residue, and a terminal 4-*O*-(1-carboxyethyl)-L-rhamnosyl group.

The mass spectra of the alditol acetate and the partially methylated alditol acetate of the (2-hydroxy-1-methylethyl)-substituted D-galactose were not in accordance with the originally proposed structure having the substituent in the 4-position [5]. The fragmentation pattern indicated instead a 6-*O*-(2-hydroxy-1-methylethyl)-D-galactose residue linked through its 4-position [3]. Thus, the corresponding non-reduced form present in the

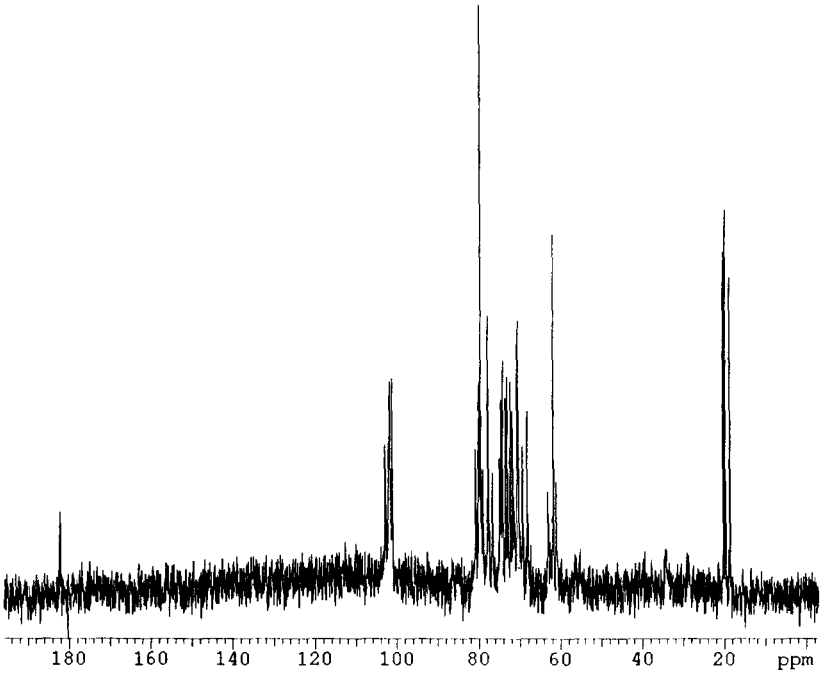

Fig. 2. 100.5-MHz ^{13}C NMR spectrum of *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain 49.

polysaccharide should be 6-*O*-(1-carboxyethyl)-D-galactose. This same sugar has also been found in *B. fibrisolvens* strain X6C61 polysaccharide [3].

The alditol acetates of the 6-*O*-(2-hydroxy-1-methylethyl)-D-galactose and the 4-*O*-

Table 1
Selected ^1H and ^{13}C NMR data obtained at 70°C for *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain 49

Residue	Chemical shifts (δ)							Coupling constants (Hz)	
	H-1	H-2	H-3	H-4	H-5	H-6	C-1	$J_{H-1,H-2}$	$^1J_{C,H}$
→ 4)-α-D-Gal *p*-(1 →	5.41	3.93	4.12	4.10			100.88	2.4	168
(4-*O*-*S*)-α-L-Rha *p*-(1 → [a]	5.18	4.05	3.93	3.39	4.33	1.30	101.22	< 2	172
→ 4)-(6-*O*-*R*)-α-D-Gal *p*-(1 → [b]	5.02	3.93	4.00	4.16			101.03	3.7	173
→ 3,4)-α-D-Glc *p*-(1 →	4.94	3.73	3.97	3.77			100.30	2.7	174
→ 4)-β-D-Glc *p*-(1 →	4.52	3.35	3.76	3.57	3.56		102.00	8.3 [c]	161
1-(*R*)-Carboxyethyl		3.89	1.32						
1-(*S*)-Carboxyethyl		4.23	1.36						

[a] 4-*O*-[(*S*)-1-Carboxyethyl]-α-L-Rha *p*-(1 →.
[b] → 4)-6-*O*-[(*R*)-1-Carboxyethyl]-α-D-Gal *p*-(1 →.
[c] Approximate values, measured from a phase sensitive H,H-COSY experiment.

(2-hydroxy-1-methylethyl)-L-rhamnose obtained from the carboxyl-reduced polysaccharide were compared on GLC–MS with those prepared from the carboxyl-reduced [8] (*S*) and (*R*) form of synthetic 6-*O*-(1-carboxyethyl)-D-galactose and 4-*O*-(1-carboxyethyl)-L-rhamnose [11]. Both the mass spectra and the retention times showed the components to be 6-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-galactose and 4-*O*-[(*S*)-2-hydroxy-1-methylethyl]-L-rhamnose. These are the reduced forms of 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose and 4-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose, which consequently are the components in the native polysaccharide.

From the ^{1}H and ^{13}C NMR spectra (Table 1) of the *O*-deacetylated polysaccharide, information on the respective sugar residues and the anomeric configurations could be deduced. Using different 1D and 2D experiments, H,H-COSY, relay and double relay H,H-COSY, most of the ^{1}H NMR signals could be assigned and the $J_{1,2}$ values determined. HMQC experiments allowed the assignment of the corresponding C-1 signals and the $^{1}J_{C,H}$ values. From a resolution-enhanced ^{1}H NMR spectrum, and the pattern of several cross-peaks in the phase-sensitive COSY spectrum, the size of the coupling constants between ring protons could be estimated. This information together with published chemical shift data for the sugars [12] allowed the assignment of the different spin-systems to specific sugar residues. The NMR data also showed that the 4-substituted D-glucose was a β-pyranoside whereas the other residues were α-pyranosides.

To identify the 4-*O*-(1-carboxyethyl)-L-rhamnose substitution position, the rhamnosyl group in the *O*-deacetylated polysaccharide was released by the use of mild acidic conditions (partial hydrolysis, procedure 1). Separation of the hydrolysis products by gel filtration on a Bio-Gel P-2 column afforded 4-*O*-(1-carboxyethyl)-L-rhamnose and a polymeric material. The 4-*O*-(1-carboxyethyl)-L-rhamnose was identified by its ^{1}H NMR spectrum. Methylation of the polymer followed by carboxyl-reduction with lithium triethylborodeuteride, hydrolysis, reduction with $NaBH_4$, and acetylation afforded the alditol acetates of 2,3,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-galactose, and 6-*O*-(2-hydroxy-1-methylethyl-*2,2-d*$_2$)-2,3-di-*O*-methyl-D-galactose in the relative proportions 2:1:1, indicating that all the monosaccharide residues were 4-substituted in the derived polysaccharide. Thus the 4-*O*-(1-carboxyethyl)-L-rhamnosyl group was linked to the 3-position in the 3,4-substituted D-glucose of the original polysaccharide.

To investigate the anomeric configuration of the disubstituted D-glucose and to which sugar residue it is linked, the *O*-deacetylated and carboxyl-reduced polysaccharide was subjected to a Smith degradation [13]. It was first oxidised with $NaIO_4$ and then reduced with $NaBH_4$, and the resulting polymer isolated by gel filtration on a column of Bio-Gel P-2. Sugar analysis of this polymer yielded only D-glucose and the ^{1}H NMR spectrum of the polymer showed only one anomeric proton signal. From the chemical shift (δ 5.07) and coupling constant ($^{3}J_{H\text{-}1,H\text{-}2}$ 3.5 Hz) of the signal, the α configuration of the disubstituted D-glucose unit was deduced. Mild acid hydrolysis of the polymer followed by separation of the hydrolysis products by gel filtration on a column of Bio-Gel P-2 yielded a glucoside. Positive FAB–MS showed an $[M + H]^+$ ion at m/z 343.2. Sugar analysis of the glucoside gave equimolar amounts of D-glucose and 1-*O*-(2-hydroxy-1-methylethyl)-D-threitol. The ^{1}H NMR spectrum showed one signal for anomeric protons at δ 5.07 with $^{3}J_{H\text{-}1,H\text{-}2}$ 4.0 Hz. These data, together with the results from the sugar

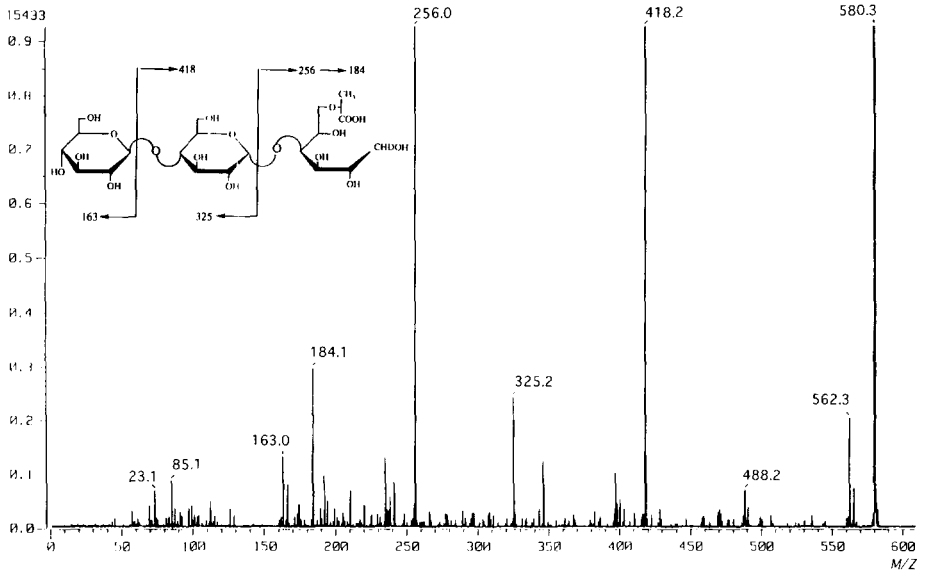

Fig. 3. Collision-induced fragment ions from the reduced trisaccharide observed in a MS/MS experiment using the $[M+H]^+$ ion at m/z 580.3, obtained by positive FABMS.

analysis, indicated that the structure of the Smith degradation product is α-D-Glc*p*-(1 → 3)-1-*O*-(2-hydroxy-1-methylethyl)-D-threitol. Thus the data from the Smith degradation and the partial hydrolysis releasing the 4-*O*-(1-carboxyethyl)-L-rhamnosyl group from the 3-position of the branched residue indicated the presence of the following sequence in the *O*-deacetylated polysaccharide:

4-*O*-[(*S*)-1-carboxyethyl]-α-L-Rha*p*-(1 → 3)-α-D-Glc*p*-(1 → 4)-

-6-*O*-[(*R*)-1-carboxyethyl]-D-Gal*p*.

In order to obtain more information on the sequence of sugar residues, a partial hydrolysis and using stronger conditions of the *O*-deacetylated polysaccharide was performed (partial hydrolysis, procedure 2). The hydrolysis products were reduced with $NaBD_4$ and separated by gel filtration to afford, among other products, a tri- and a tetra-saccharide. The matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrum of the trisaccharide showed an $[M+Na]^+$ ion at m/z 602, indicating that the deuterium-reduced trisaccharide molecular weight was 579. The positive FABMS spectrum of the deuterium-reduced trisaccharide showed an $[M+H]^+$ ion at 580.3. According to the measured molecular weight of the deuterium-reduced trisaccharide the non-reduced form is composed of two hexoses and one 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose residue. The high-energy collision-induced fragmentation mass spectrum (Fig. 3) of the ion at m/z 580.3, using He as the collision gas, indicated two hexose residues and 6-*O*-[(*R*)-1-carboxyethyl]-D-galactitol-*1-d* at the reduced end [14]. Sugar analysis of the trisaccharide yielded only glucose, indicating that glucose is the

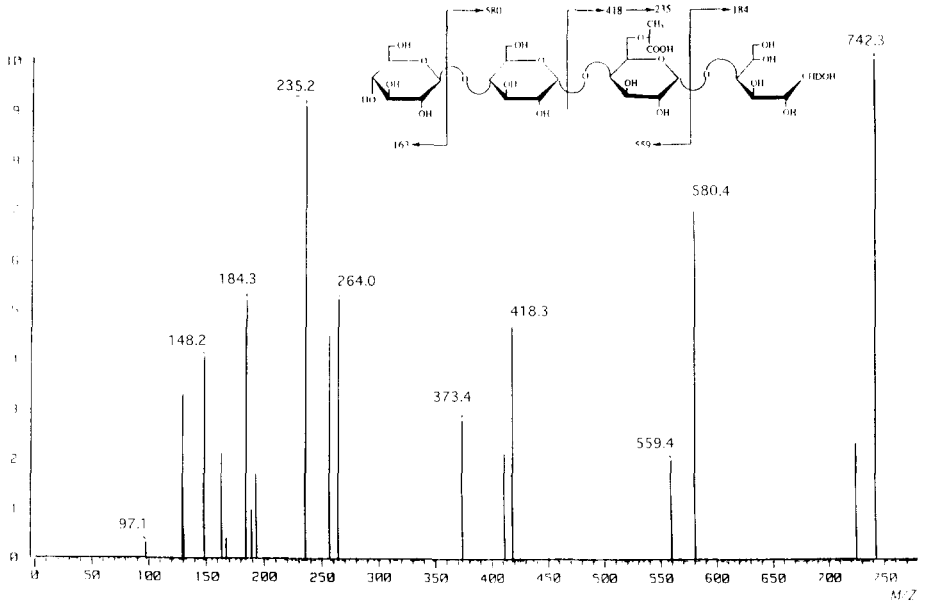

Fig. 4. Collision-induced fragment ions from the reduced tetrasaccharide observed in a MS/MS experiment using the $[M+H]^+$ ion at m/z 742.3, obtained by positive FABMS.

only neutral monosaccharide present in the molecule. The ^{1}H NMR spectrum of the reduced trisaccharide showed signals from an α- and a β-anomeric proton, according to their chemical shifts (δ 5.08 and 4.51) and coupling constants ($^3J_{H\text{-}1,H\text{-}2}$ 4.0 and 8.0 Hz, respectively). These data, together with the results from the Smith degradation, implied that the reduced trisaccharide has the following structure.

β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-6-*O*-[(*R*)-1-carboxyethyl]-D-galactitol-*1*-*d*.

The MALDI-TOF spectrum of the tetrasaccharide showed an $[M + Na]^+$ ion at m/z 764 and a minor peak at 780, corresponding to $[M + K]^+$, indicating that the reduced tetrasaccharide had a molecular mass of 741. According to this the non-reduced form is composed of three hexose residues and the 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose residue, and corresponds to one repeating unit of the linear polysaccharide chain. The collision-induced fragmentation of the $[M + H]^+$ ion at m/z 742.3, using He as the collision gas, indicated a hexitol at the reduced end, to which the 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose residue was linked (Fig. 4) [14]. Acid hydrolysis of the reduced tetrasaccharide followed by acetylation and GLC–MS analysis afforded D-galactitol-*1*-*d* hexaacetate and two penta-*O*-acetylhexopyranoses, thus indicating the presence of D-galactose in the tetrasaccharide reducing end.

The ^{1}H NMR spectrum of the reduced tetrasaccharide showed signals from three anomeric protons. Their chemical shifts (δ 5.14, 4.95, and 4.53) and $^3J_{H\text{-}1,H\text{-}2}$ values (3.8, 3.8, and 7.9 Hz, respectively) implied one β- and two α-glycosidic linkages. As is indicated by the trisaccharide structure, the glucosyl group at the non-reducing end is

Table 2
Observed inter-residue NOEs in the NOESY spectrum for anomeric protons in the *O*-deacetylated polysaccharide

Anomeric proton	NOE (δ and proton)
5.41 (α-D-Gal*p*)	3.57 (H-4; β-D-Glc*p*)
5.18 ([4-*O*-*S*]-α-L-Rha*p*) [a]	3.96 (H-3; $\rightarrow$ 3,4-α-D-Glc*p*)
5.02 ([6-*O*-*R*]-α-D-Gal*p*) [b]	4.10 (H-4; α-D-Gal*p*)
4.94 (α-D-Glc*p*)	4.16 (H-4; [6-*O*-*R*]-α-D-Gal*p* [b])
4.52 (β-D-Glc*p*)	3.77 (H-4; α-D-Glc*p*) [c]

[a] 4-*O*-[(*S*)-1-Carboxyethyl]-α-L-Rha*p*.
[b] 6-*O*-[(*R*)-1-Carboxyethyl]-α-D-Gal*p*.
[c] Coincides with intra-residue NOE between H-1 and H-3.

β-linked and thus the 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose residue is α-linked to the galactitol residue. All the above data indicated the reduced tetrasaccharide structure as:

$$\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-6-}O\text{-}[(R)\text{-1-carboxyethyl}]\text{-}$$
$$\text{-}\alpha\text{-D-Gal}p\text{-}(1 \rightarrow 4)\text{-D-galactitol-}1\text{-}d.$$

Sequence information was also obtained by the inter-residue NOEs between the anomeric proton of one residue and the proton on the linkage carbon of the next residue, observed as cross-peaks in a NOESY spectrum. The inter-residue NOEs obtained for all disaccharide elements in the repeating unit of the *O*-deacetylated polysaccharide are shown in Table 2. An NOE between H-4 of the 4-*O*-[(*S*)-1-carboxyethyl]-L-rhamnosyl group and H-2 of the carboxyethyl group allowed its assignment.

These results, together with other NMR data (Table 1) and the structure of the tri- and tetra-saccharides, showed the structure of the repeating unit of the polysaccharide:

$\rightarrow$ 4)-β-D-Glc*p*-(1 $\rightarrow$ 4)-α-D-Glc*p*-(1 $\rightarrow$ 4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1 $\rightarrow$ 4)-α-D-Gal*p*-(1 $\rightarrow$

3
↑
1

4-*O*-[(*S*)-1-carboxyethyl]-α-L-Rha*p*.

The substitution position of the major part of the *O*-acetyl groups was determined from a comparison of ^{1}H NMR and 2D-COSY spectra of the native polysaccharide with those of the *O*-deacetylated polysaccharide. The chemical shifts for the H-1 to H-3 signals of the α-D-Gal*p* residue were δ 5.44, 4.23, and 5.13, respectively. This is in agreement with the expected shifts for a 3-*O*-acetylation of this residue according to previous studies of *O*-acetylated monosaccharides [15]. Due to several overlapping minor signals in the ^{1}H NMR spectrum of the native polysaccharide, the positions of the remaining *O*-acetyl groups could not be determined.

The capsular polysaccharide made by *B. fibrisolvens* strain 49 contains several interesting molecular features. Two acidic sugars, thus far unique to the genus *Butyrivibrio*, namely 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose and 4-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose, are encountered within the pentasaccharide repeating unit. The former occurs as part of the backbone of the polymer made by both strain 49 and X6C61, the only other strain for which data are available [3]. It has been detected, however, in

hydrolyzates from roughly half of the *Butyrivibrio* strains screened to date [1]. The latter compound occurs as a substituent to O-3 of the α-D-Glc*p* backbone residue in strain 49, but in contrast is only rarely found in the polysaccharides made by other *Butyrivibrio* strains [1].

Though there are many other differences between the structures of the polysaccharides made by strains 49 and X6C61, they do share a common trisaccharide epitope:

→ 4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1 → 4)-α-D-Gal*p*-(1 → 4)-

-β-D-Glc*p*-(1 →

However, the 3-position of the α-D-Gal*p* residue of this trisaccharide is *O*-acetylated in strain 49, while the 2-position contains α-L-Rha*p* in strain X6C61 [3]. *O*-Acetyl groups are also present in the capsular polysaccharide of strain X6C61, but are found at different locations.

Interestingly, all of the linkages along the backbone of the polymers made by both strains 49 and X6C61 are 1 → 4. This is despite the fact that a great deal of the ingested plant polymeric materials which serve as substrates for ruminant microorganisms, such as cellulose, starch, xylans, and pectins, are themselves rich in 1 → 4 linkages. We continue to believe and propose that 1-carboxyethylation, *O*-acetylation, and other structural modifications are all strategies adopted by *B. fibrisolvens* to confer resistance to hydrolysis of its polysaccharides by the multitude of hydrolytic enzymes found within the rumen. We are continuing our studies with other strains in order to work out the major structural epitopes found in these interesting organisms.

3. Experimental

General methods.—Concentrations were performed under reduced pressure at 40°C, or by flushing with N_2. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionization detector was used. Separation of the alditol acetates and the partially methylated alditol acetates was performed on an HP-5 fused-silica capillary column, using a temperature program, from 140°C (3 min) to 250°C at 3°C min^{-1}. GLC–MS was performed on a Hewlett–Packard 5970 MSD instrument, using the column and conditions mentioned above. Methylation analysis was performed as described [16] and the methylated products were recovered using a Sep-Pak C-18 cartridge [17]. NMR spectra were recorded for solutions in D_2O at 70°C, using a Varian VXR 400 instrument. The samples were passed through a column of Dowex 50 (Na^+ form) prior to NMR analysis. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropionate-d_4 (TSP, δ_H 0.00) and acetone (δ_C 31.07) as internal references. 2D (COSY, relayed COSY, NOESY, and HMQC) experiments were performed according to standard pulse sequences available in the Varian software. A 90° pulse was used in the correlation experiments, and in the NOESY experiment a mixing time of 0.3 s. FABMS spectra were recorded on a JEOL JMS-SX/SX-102A tandem mass spectrometer by bombardment of the samples (dissolved in a glycerol matrix) with Xe atoms of average translational energy 6 keV. The mass spectrometer was operated at an accelerating

voltage of 10 kV. Tandem mass spectrometry was performed using the first two sectors (B_1E_1), comprising MS-1, to select the precursor ions and the second mass spectrometer (B_2E_2), comprising MS-2, to analyse the product ions. A resolution of approximately 3.000 was used to separate the ^{12}C peak of the $[M + H]^+$ precursor ions. Helium was used as the collision gas at a pressure sufficient to attenuate the precursor ion beam by approximately 50%. The MALDI-TOF spectra were recorded on a Linear LDI 1700XP instrument, using 2,5-dihydroxybenzoic acid as matrix. For better precision of the ions a starch hydrolysate was used as internal standard.

Pyridine–acetate buffer (0.1 M, pH 6.8) was used as mobile phase for all gel filtrations. The eluate was monitored using a Knauer differential refractometer and all fractions were checked by ^1H NMR spectroscopy or MALDI-TOF spectrometry.

Organism, growth conditions, and isolation of extracellular polysaccharides.—*Butyrivibrio fibrisolvens* strain 49 was grown on 1% D-glucose under anaerobic conditions on the defined medium of Cotta and Hespell [18] supplemented with 0.3% Trypticase. Cells were removed from stationary-phase cultures by centrifugation, and crude extracellular polysaccharide was phenol-extracted, dialysed, and lyophilized as previously described [2].

Purification of the native polysaccharide.—The crude polysaccharide was further purified by anion-exchange chromatography. In a typical run, crude polysaccharide (160 mg) was applied to a column (1.5 × 12 cm) of DEAE-Sepharose, which was irrigated first with water (40 mL) and then with a linear gradient of aqueous NaCl (0–1 M, 200 mL). The fractions were monitored by reaction with phenol and H_2SO_4. The acidic polysaccharide (75 mg) was recovered after dialysis and freeze-drying.

O-*Deacetylation of polysaccharide.*—Purified native polysaccharide (50 mg) was treated with aq 12.5% NH_4OH (5 mL) at room temperature for 16 h, and the resulting material dialyzed and freeze-dried to give *O*-deacetylated polysaccharide (33 mg).

Carboxyl reduction of O-*deacetylated polysaccharide.*—Reduction was performed according to Taylor et al. [8] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and $NaBH_4$ or $NaBD_4$ as reducing agent. The procedure was repeated twice in order to obtain a more complete reduction. The reaction was checked by ^1H NMR spectroscopy.

Partial hydrolysis.—Procedure 1. O-Deacetylated polysaccharide (22 mg) was treated with 50 mM aqueous CF_3CO_2H (5 mL) at 80°C for 4 h whereafter the solution was freeze-dried. The products were isolated by gel filtration on a column (1.6 × 75 cm) of Bio-Gel P-2. The linear polysaccharide was eluted in the void volume.

Procedure 2. The same conditions as in procedure 1 were used but for a longer reaction time (22 h). The reaction mixture was freeze-dried and the resulting oligosaccharides were dissolved in M NH_4OH (2 mL) and reduced with $NaBD_4$. Gel filtration on a column of Bio-Gel P-2 gave several fractions which were checked by ^1H NMR spectroscopy and MALDI-TOF spectrometry. A tri- and a tetra-saccharide (1.0 and 1.5 mg, respectively) could be isolated among other products.

Smith degradation.—Carboxyl-reduced polysaccharide (14 mg) was treated with $NaIO_4$ (64 mg) in NaOAc buffer (0.1 M, pH 3.7) at 4°C for 18 h, excess of $NaIO_4$ destroyed by addition of ethylene glycol (50 μL), and the product reduced with $NaBH_4$ (15 mg in 1.5 mL M NH_4OH), and then dialyzed against distilled water and freeze-dried.

The ^{1}H NMR spectrum indicated incomplete oxidation, so the above process was repeated to obtain a more completely oxidized product. Sugar analysis of the polymeric product obtained after dialysis (3.5 mg) gave the acetates of erythritol, threitol, 1-*O*-(2-hydroxy-1-methylethyl)threitol, and glucitol. The product was treated with 0.5 M CF_3CO_2H (0.5 mL) at room temperature for 20 h, the solution was freeze-dried, the products were separated by gel filtration on a column of Bio-Gel P-2, and the fractions checked by FABMS and ^{1}H NMR spectroscopy.

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Council for Forestry and Agricultural Research, and The International Program in the Chemical Sciences (IPICS), Uppsala, Sweden.

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