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Structural studies of the extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61

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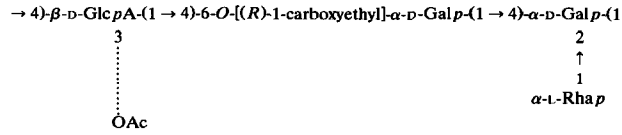
ABSTRACT

The capsular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61 has been investigated using NMR spectroscopy, mass spectrometry, methylation analysis, and partial acid hydrolysis as the main methods. The polysaccharide is composed of hexasaccharide repeating units having the following structure.



The polysaccharide also contains *O*-acetyl groups, of which ~ 70% are substituted to O-3 of the β-D-Glc*p*A residue.

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* evidently 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], 4-*O*-[1-

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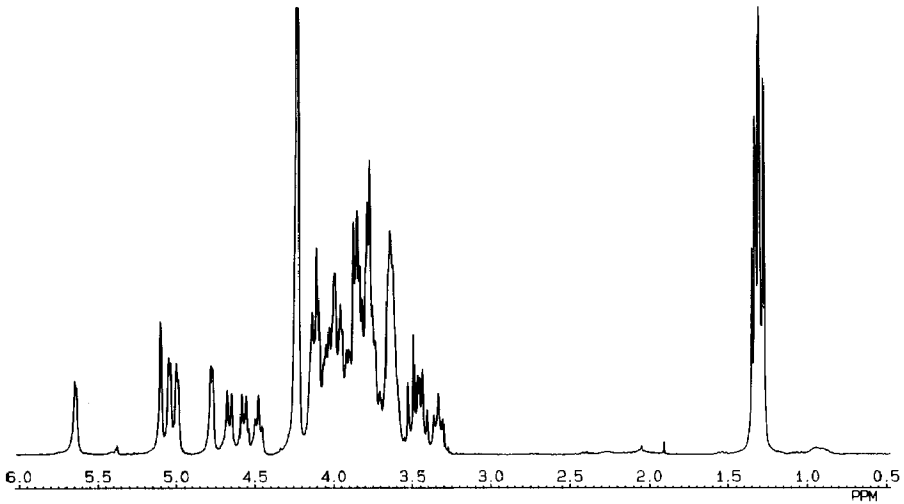


Fig. 1. 270-MHz ^{1}H NMR spectrum of the *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61.

carboxyethyl]-L-rhamnose[3], and others in a strain-specific manner[1]. For example, out of more than forty strains examined, only the extracellular polysaccharide designated as EPS-II from strain X6C61 contained L-iduronic acid[4]. Though quite commonly found in such glycosaminoglycans as heparin[5], L-iduronic acid is rarely encountered in microbial polysaccharides. A subsequent study identified 6-deoxy-D-talose as a constituent of EPS-I[6], a neutral polymer comprising less than 10% of the total polysaccharide produced by strain X6C61.

No information on the structural characteristics of these interesting polysaccharides has been reported to date. We have thus initiated studies to define more precisely the structure of the repeating unit of the major extracellular polysaccharide (EPS-II) produced by strain X6C61 and report herein our results.

RESULTS AND DISCUSSION

The crude capsular material was prepared as described[4] 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, together corresponding to three protons. The polysaccharide was *O*-deacetylated and the simplified ^{1}H and ^{13}C NMR spectra (Figs. 1 and 2) of the product showed signals from six anomeric proton and carbon atoms, verified by a C,H-COSY spectrum, which indicated a hexasaccharide repeating unit. The chemical shifts of these signals and coupling constants, $^3J_{H\text{-}1,H\text{-}2}$ and $^1J_{C,H}$ (Table I), indicated that the repeating unit consisted of four α- and two β-pyranosyl residues. The ^{13}C NMR spectrum also contained signals for two carbonyl carbon atoms at $\delta \sim 175$ and one

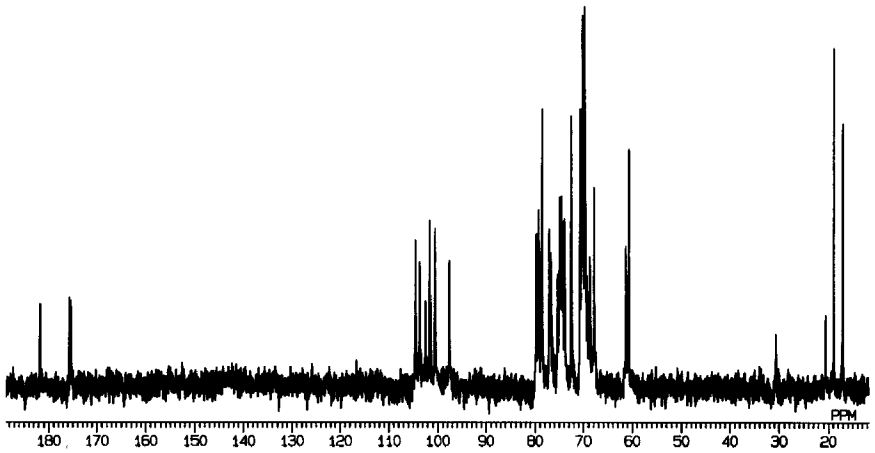


Fig. 2. 67.8-MHz ^{13}C NMR spectrum of the *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61.

at $\delta \sim 180$, indicating the presence of L-iduronic acid and (1-carboxyethyl)-D-galactose, which have been found earlier[4,7], and an additional uronic acid.

In order to facilitate the sugar and methylation analysis, the polysaccharide was carboxyl-reduced[8] using sodium borodeuteride as the reducing agent. The procedure had to be repeated in order to obtain a higher yield in the reduction. Hydrolysis of the product, reduction with sodium borohydride, and acetylation yielded the acetates of 1,6-anhydroidopyranose-*6,6-d*$_2$, rhamnitol, galactitol, glucitol (with $\sim 40\%$-*6,6-d*$_2$), iditol-*6,6-d*$_2$, and a [2-(1-hydroxy)propyl-*1,1-d*$_2$]hexitol in the relative proportions 0.7 : 1.0 : 1.0 : 1.6 : 0.1 : 0.8, as revealed by GLC–MS. The

TABLE I

^1H and ^{13}C NMR data obtained at 70°C for *O*-deacetylated extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61

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 → 2 ↑	5.66	3.96	4.07	4.13	3.99	n.d. [a]	97.62	3.0	176
α-L-Rha*p*-(1 →	5.09	4.10	3.80	3.47	3.80	1.27	102.29	n.r. [b]	173
→ 4)-(6-*O*-R)-α-D-Gal*p*-(1 → [c]	4.96	3.92	3.99	4.24	4.50	~ 3.62	100.42	3.5	172
→ 4)-α-L-Ido*p*A-(1 →	4.94	3.62	4.01	4.10	4.74		101.54	2.5	172
→ 4)-β-D-Glc*p*A-(1 →	4.64	3.45	3.60	3.75	3.77		104.34	7.5	163
→ 4)-β-D-Glc*p*-(1 →	4.55	3.34	3.76	3.75	3.64	n.d. [a]	103.41	7.0	164
(R)-1-Carboxyethyl		4.08	1.32						

[a] n.d., Not determined. [b] n.r., Not resolved, $\nu_{1/2} = \sim 4$ Hz. [c] → 4)-6-*O*-[(*R*)-1-Carboxyethyl]-α-D-Gal*p*-(1 →.

TABLE II

Methylation analysis of carboxyl-reduced and partially hydrolysed extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61

Sugar [a]	t_R [b]	Detector response (%) [c]		
		A	*B*	*C*
1,2,3,5,6-Galol-*1-d*				13.1 [d]
2,3,4-Rha	0.49	13.9	8.3	
2,3,4,6-Glc				20.5
2,3,6-Gal	1.99	–	11.9	
2,3,6-Glc	2.09	32.7	31.4	7.3 [e]
2,3,6-Ido	2.22	13.4	20.2	11.3 [e]
3,6-Gal	3.23	19.6	10.4	
2,3-(Lac)-Gal	4.47	20.4	17.7	21.8

[a] 2,3,4-Rha, 2,3,4-tri-*O*-methyl-L-rhamnose, etc.; (Lac)-Gal, 6-*O*-[2-(1-methoxy)propyl]-D-galactose. [b] Retention time of the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a DB-225 fused-silica capillary column at 200°C. [c] *A*, Carboxyl-reduced polysaccharide; *B*, carboxyl-reduced, partially hydrolysed polysaccharide; *C*, reduced ($NaBD_4$) pentasaccharide. [d] Part of this sugar was obtained as the 3,4,5-tri-*O*-Ac-1,2,6-tri-*O*-Me-D-Galol-*1-d* probably due to a boric ester (65%). [e] Obtained as the *6,6-d*$_2$-derivatives.

occurrence of glucitol-*6,6-d*$_2$ in the sugar analysis indicated the presence of glucuronic acid in the native polysaccharide. The equilibrium between idose and its 1,6-anhydropyranose derivative, established in the dilute aqueous acid used for the hydrolysis, yielded ca. 90% of the latter sugar. The absolute configurations of the L-iduronic acid and the (1-carboxyethyl)-D-galactose were determined earlier[4,7], and now the absolute configurations of the other sugars have been determined by GLC of the trimethylsilylated (+)-2-butyl glycosides[9] and shown to be L-rhamnose, D-galactose, D-glucose, and D-glucuronic acid.

Methylation analysis[10] of the carboxyl-reduced polysaccharide (Table II, column *A*) demonstrated that the L-rhamnopyranosyl is terminal, the D-galactopyranosyl residue is branched and linked through O-2 and O-4, and all the other sugar residues are linked through O-4.

The mass spectra of the alditol acetate and the partially methylated alditol acetate of the 2-(1-hydroxy)propyl-substituted D-galactose (Figs. 3 and 4) were not in accordance with our previously suggested structure with the hydroxypropyl substituent in the 4-position[7]. The fragmentation pattern suggested instead a 6-*O*-[2-(1-hydroxy)propyl]-D-galactose linked through its O-4. The alditol acetate of the natural compound was therefore compared by GLC–MS with those prepared from the (*S*)- and (*R*)-form of synthetic 6-*O*-[2-(1-hydroxy)propyl]-D-galactose[11]. Both the mass spectrum and the retention time showed the component to be 6-*O*-[(*R*)-2-(1-hydroxy)propyl]-D-galactose. This is the reduced form of 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose, which consequently must be the component in the native polysaccharide.

From the ^1H and ^{13}C NMR spectra (Table I) of the *O*-deacetylated polysaccharide, information on the respective sugar residues and the anomeric configurations

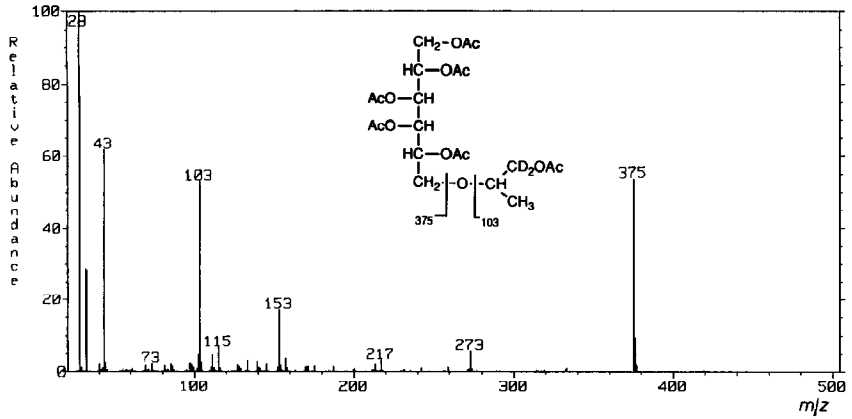


Fig. 3. Mass spectrum and the most abundant fragments for the alditol acetate of 6-*O*-[(*R*)-2-(1-hydroxy)propyl-*1,1-d*$_2$]-D-galactose.

could be deduced. Using different 1D and 2D experiments, 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 $^1J_{C,H}$ values. From the pattern of the cross-peaks in the COSY spectrum, the size of the coupling constants for couplings 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 D-glucose and the D-glucuronic acid residues were β-pyranosides, whereas the other residues were α-pyranosides. The anomeric configuration of the L-iduronic acid residue was deduced from the coupling constants (Table I), and the proton resonances were in agreement with previously published data[13] on 4-linked α-L-iduronic acid residues. From the $^1J_{C,H}$ value and

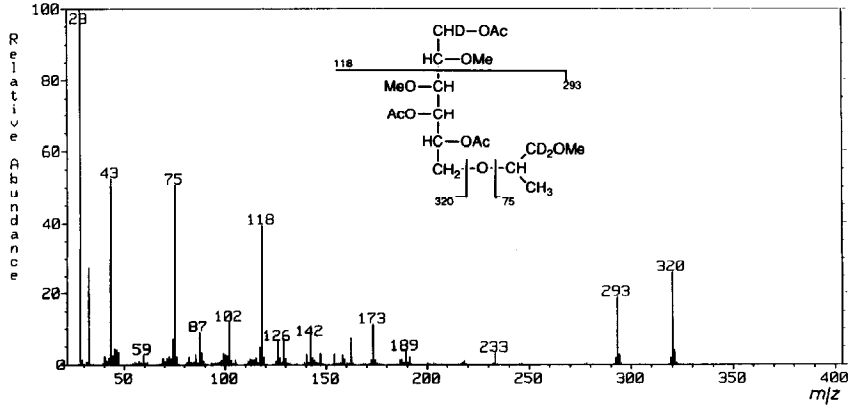


Fig. 4. Mass spectrum and the most abundant fragments for the alditol acetate of 6-*O*-[(*R*)-2-(1-methoxy)propyl-*1,1-d*$_2$]-2,3-di-*O*-methyl-D-galactose.

the size of the three-bond couplings ($J_{1,2}$ 2.5, $J_{2,3} \sim 5$, $J_{3,4} \sim 6$, and $J_{4,5} \sim 4$ Hz) between the ring protons, it was evident that the $^1C_4$ conformation[13,14] of the L-iduronic acid residue was highly populated in the polymer.

In order to obtain information on the sequence of the sugar residues, several different partial acid hydrolyses were performed. Treatment of the reduced polysaccharide with 50 mM trifluoroacetic acid at 80°C for 16 h followed by gel filtration yielded a polymeric material and free L-rhamnose. Methylation analysis of the polymeric material (Table II, column *B*) showed that the 2,4-linked residue in the polysaccharide was converted into a 4-linked residue, indicating that the α-L-rhamnopyranosyl group is linked to the 2-position of the branching D-galactopyranosyl residue in the polysaccharide.

To isolate oligosaccharide fragments from the main chain of the polysaccharide, two different partial acid hydrolyses were performed, in which the native polysaccharide was hydrolysed under stronger acidic conditions (0.1 M trifluoroacetic acid at 100°C for 16 and 2 h, respectively) than in the experiment described above. The products in the first experiment were carboxyl- and carbonyl-reduced with sodium borodeuteride and then separated by gel filtration on Bio-Gel P-2. A fraction was obtained which contained a mixture of a di- and a tri-saccharide, and another fraction with only the disaccharide. A FAB mass spectrum, obtained in the negative mode, of the first fraction had an $[M-1]^-$ ion (m/z 568) corresponding to a trisaccharide consisting of a hexose with two deuterium atoms, the 6-*O*-[2-(1-hydroxy)propyl]-D-galactose with two deuterium atoms, and a hexitol containing one deuterium atom. The spectrum of the disaccharide fraction showed an $[M-1]^-$ ion at m/z 406 (a difference of 162 from the trisaccharide), which indicates a disaccharide consisting of the first two sugars of the trisaccharide with one of them in the reduced form. Sugar analysis of the mixture gave D-glucose-*6,6-d*$_2$, 6-*O*-[(*R*)-2-(1-hydroxy)propyl-*1,1-d*$_2$]-D-galactose, 6-*O*-[(*R*)-2-(1-hydroxy)propyl-*1,1-d*$_2$]-D-galactitol-1-*d*, and D-galactitol-1-*d* in the relative proportions 1.7 : 1.1 : 0.5 : 1.0. The ^{1}H NMR spectrum of the disaccharide showed a signal at δ 4.53 (*J* 7 Hz) and that of the trisaccharide two signals at δ 5.10 (*J* 3 Hz) and 4.62 (*J* 7 Hz) for anomeric protons, indicating that, in the native polysaccharide, the D-glucuronic acid has the β configuration and the 6-*O*-(1-carboxyethyl)-D-galactose has the α configuration. These results indicate that the trisaccharide element **1** is a part of the polysaccharide repeating unit.

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

1

The products in the third experiment were separated by gel filtration on Bio-Gel P-2 and yielded a fraction which contained a pentasaccharide. FABMS in the negative mode showed that the pentasaccharide fraction actually consisted of two pentasaccharides ($[M-1]^-$, m/z 927 and 969). Each of these pentasaccharides contained two hexoses, two uronic acids, and 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose; one contained, in addition, an *O*-acetyl group. Using a B/E-linked scan of the

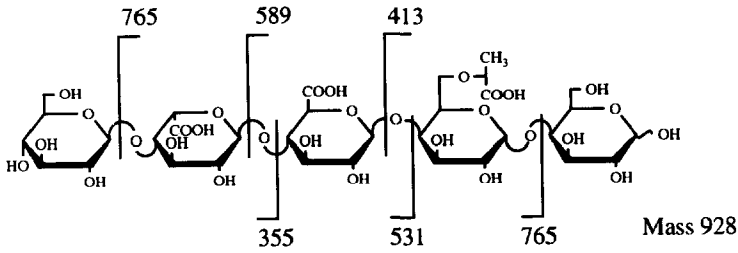


Fig. 5. Fragment ions from the pentasaccharide observed in a B/E-linked scan experiment using the $[M-1]^-$ ion obtained by negative FABMS.

m/z 927 ion, a series of fragment ions at *m/z* 765, 531, and 355 and another series of ions at *m/z* 589 and 413 were obtained (Fig. 5). In addition to these ions, other ions derived from these by the loss of water were observed. The first series of ions was generated from the nonacetylated pentasaccharide after the loss of a hexose, 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose, and a uronic acid, respectively, leaving a fragment containing a hexose and a uronic acid. The other series is derived from the loss of a disaccharide fragment consisting of a hexose and a uronic acid, and then another uronic acid. In the ^1H NMR spectrum of the carboxyl-reduced, non-acetylated pentasaccharide, signals for anomeric protons were observed at δ 5.11, *J* 3.3; 4.82, *J* 4.8; 4.70, *J* 7.7; and 4.50, *J* 7.7 Hz.

These data, in combination with methylation analysis of the carboxyl-reduced pentasaccharide (Table II, column *C*) showing the two end-groups, and with the results obtained for the trisaccharide fragment **1**, indicate that the pentasaccharide has the following structure:

β-D-Glc*p*-(1 → 4)-α-L-Ido*p*A-(1 → 4)-β-D-Glc*p*A-(1 → 4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1 → 4)-D-Gal

2

Sequence information was also obtained by the inter-residue NOEs, i.e., between the anomeric proton and the proton on the linkage carbon observed in a NOESY spectrum. Table III shows the inter-residue NOEs obtained for all disaccharide elements in the repeating unit. These results, together with other

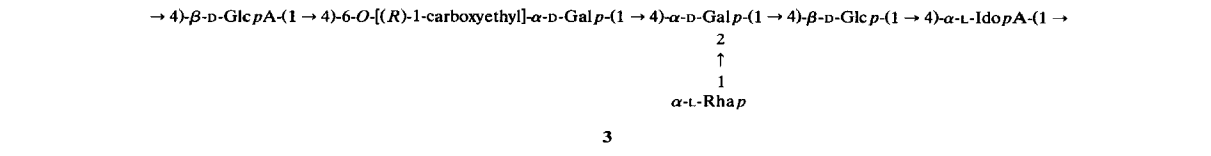
TABLE III

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

Anomeric proton	NOE [δ and (proton; residue)]
5.66 (α-D-Gal*p*)	3.75 (H-4; β-D-Glc*p*)
5.09 (α-L-Rha*p*)	5.66 and 3.96 (H-1 and H-2; α-D-Gal*p*)
4.96 ([6-*O*-R]-α-D-Gal*p*) [a]	4.13 (H-4; α-D-Gal*p*)
4.94 (α-L-Ido*p*A)	3.75 (H-4; β-D-Glc*p*A)
4.64 (β-D-Glc*p*A)	4.24 (H-4; [6-*O*-R]-α-D-Gal*p*)
4.55 (β-D-Glc*p*)	4.74 and 4.10 (H-5 and H-4; α-L-Ido*p*A)

[a] 6-*O*-[(*R*)-1-Carboxyethyl]-α-D-Gal*p*.

NMR data (Table I) and the structure of pentasaccharide **2**, give the structure of the repeating unit **3** of the polysaccharide:



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The position of the major part (70%) of the *O*-acetyl group was determined from a comparison of 1D- and 2D-COSY ^{1}H NMR spectra of the native polysaccharide with those of the *O*-deacetylated polysaccharide. The chemical shifts for the H-1 to H-4 signals of the β-D-Glc*p*A residue were δ 4.75, 3.60, 5.05, and 3.93, 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 FAB data obtained from the B/E-linked scan of the *O*-acetylated pentasaccharide was also consistent with the ^{1}H NMR data in assigning most of the acetylation to the 3-position of the β-D-Glc*p*A residue. The *m/z* 969 ion generated fragment ions of *m/z* 765 and 589, indicating that these fragments must contain the *O*-acetyl group. The presence of the disaccharide fragment (*m/z* 355), consisting of a hexose and a uronic acid, showed that the *O*-acetyl group was not located on this disaccharide fragment.

The structure of the *B. fibrisolvens* strain X6C61 extracellular polysaccharide contains several unusual features. Its hexasaccharide repeating unit has three different acidic sugars and one of these, namely 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose, has not previously been reported as a component of any naturally occurring material. The other two acidic sugars, D-glucuronic and L-iduronic acids, are C-5 epimers. This strongly suggests that strain X6C61 of *B. fibrisolvens* possesses a bacterial C-5 epimerase enzyme, perhaps analogous to that which catalyses the same reaction in mammalian systems during the biosynthesis of heparan sulfate[5].

Previous studies of extracellular polysaccharides made by other strains of *B. fibrisolvens* revealed several instances wherein a second pair of C-5 epimers (namely, D-galactose and L-altrose) were present in the same material[16]. It is thus likely that an additional and possibly unique C-5 epimerase enzyme is possessed by these specific strains of *B. fibrisolvens*. Similarly, other 1-carboxyethyl-substituted sugars of identified [4-*O*-(1-carboxyethyl)-L-rhamnose][3] and as yet unidentified structure[1] have been reported to be constituents of other extracellular polysaccharides made by specific strains of *B. fibrisolvens*. These unusual structural features probably result from a variety of specific modification enzymes acting on the completed polysaccharide chains. The function of these modified sugars may be to prevent recognition and subsequent enzymic hydrolysis from the multitude of exo- and endo-glycosidases present in the gastrointestinal tract. We also demonstrated,

in the present study, the presence of an *O*-acetyl group on the 3-position of the Glc*p*A residue in the repeating unit of X6C61. Thus, epimerisation, 1-carboxyethylation, and *O*-acetylation all appear to be strategies adopted by *B. fibrisolvens* for the modification of its extracellular polysaccharides. We can infer from our structural studies to date that a variety of novel enzymes with unexplored potential for commercial and investigational use are present in this unusual species.

EXPERIMENTAL

General methods.—Evaporations were performed under diminished pressure at 40°C (bath), or by flushing with air. For GLC, a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed on HP-5 and DB-225 fused-silica capillary columns, using temperature programs, from 200°C (5 min) to 250°C at 5°C min^{-1} and from 190°C (1 min) to 230°C at 8°C min^{-1}, respectively. Partially methylated alditol acetates were analysed on the same columns, using temperature programs from 185°C (1 min) to 250°C at 3°C min^{-1} and from 190°C (1 min) to 230°C at 3°C min^{-1}. GLC–MS was performed on a Hewlett–Packard 5970 MSD instrument, using the columns described above. Methylation analysis was performed as described[10] and the methylated products were purified by reversed-phase chromatography on Sep-Pak C_{18}-cartridges[17]. NMR spectra of solutions in D_2O were recorded at 70°C, using a JEOL GSX-270, GX-400, or EX-400 instrument. The samples were passed through a column of Dowex 50 (Na^+) prior to NMR analysis. Chemical shifts were reported in ppm, using sodium 3-(trimethylsilyl)propanoate-d_4 (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) as internal references. Difference (HOHAHA) and 2D (COSY, relayed COSY, NOESY, and HMQC) experiments were performed according to standard pulse sequences available in the JEOL software. A 90° mixing pulse was used in the correlation experiments, and in the NOESY experiment a mixing time of 0.22 s. FABMS was performed with a JEOL SX-102 mass spectrometer and a Xe source, using a mixture of triethanolamine : tetramethylurea as matrix. The gel filtrations were monitored using a differential refractometer and all fractions checked by ^1H NMR spectroscopy.

Organism, growth conditions, and isolation of extracellular polysaccharides.—*B. fibrisolvens* strain X6C61, originally isolated by N.O. van Gylswick, National Chemical Research Laboratory, Pretoria, Republic of South Africa, was anaerobically grown to stationary phase on 1% D-glucose in the chemically defined medium of Cotta and Hespell[18] as previously described[1]. Crude extracellular polysaccharides were isolated from concentrated, dialysed culture supernatants that were phenol-extracted[19], redialysed against water, and lyophilised.

Purification of the native polysaccharide.—The crude polysaccharide (200 mg) was further purified on a column (3 × 40 cm) of DEAE-Sepharose, which was irrigated first with water (250 mL) and then with a linear gradient of aq NaCl (0–1 M, 1 L). The fractions were monitored by the anthrone reagent[20]. The acidic polysaccharide (96 mg) was recovered after dialysis and freeze-drying.

O-Deacetylation of polysaccharide.-The polysaccharide (15 mg) was treated with 0.1 M aq NaOH (5 mL) at room temperature for 4 h. The solution was neutralised with Dowex 50 (H^+), dialysed, and freeze-dried to give the *O*-deacetylated polysaccharide (11 mg).

Carboxyl reduction of O-deacetylated polysaccharide.—Reduction was performed according to Taylor et al.[8], using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and $NaBH_4$ or $NaBD_4$ as reducing agent. The procedure had to be repeated in order to obtain complete reduction of the D-glucuronic acid residue. The reaction was checked by sugar analysis and ^1H NMR spectroscopy.

Partial acid hydrolysis.—Procedure 1. Carboxyl-reduced polysaccharide (5 mg) was treated with 50 mM CF_3CO_2H (2 mL) at 80°C for 16 h and then concentrated to dryness. The products were purified by gel filtration on a column (90 × 1.5 cm) of Bio-Gel P-2, which was irrigated with water containing 1% of 1-butanol. A polymeric material (3 mg) was eluted in the void volume, and later L-rhamnose (< 1 mg).

Procedure 2. Native polysaccharide (15 mg) in 0.1 M CF_3CO_2H (3 mL) was kept at 100°C for 16 h. The solution was evaporated to dryness and the product dissolved in water (1 mL). Fractionation by gel filtration on a column (90 × 1.5 cm) of Bio-Gel P-2, which was irrigated with water containing 1% of 1-butanol, gave a fraction containing a mixture of a di- and a tri-saccharide (2 mg), and a fraction containing a disaccharide (2 mg).

Procedure 3. Native polysaccharide (20 mg) in 0.1 M CF_3CO_2H (3 mL) was kept at 100°C for 2 h. The solution was evaporated to dryness and the product dissolved in water (0.5 mL). Fractionation by gel filtration on a column (100 × 1.5 cm) of Bio-Gel P-2 (Superfine > 400 mesh; packed under elevated pressure), which was irrigated with pyridinium acetate buffer (0.05 M, pH 5.5), gave two fractions containing polymeric material (5.6 mg) and a pentasaccharide fraction (4.2 mg), respectively. Part of the pentasaccharide fraction (2.5 mg) was carboxyl-reduced[8] with $NaBD_4$ (5 mg) in water (1 mL) for 2 h. After conventional workup, the reduced and *O*-deacetylated pentasaccharide was subjected to ^1H NMR spectroscopy and methylation analysis.

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