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Structural studies of the extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain CF3

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Abstract

The structure of the *Butyrivibrio fibrisolvens* strain CF3 capsular polysaccharide has been investigated mainly by sugar and methylation analyses, Smith degradation, NMR spectroscopy, and mass spectrometry. The results indicate that the polysaccharide is composed of pentasaccharide repeating units having the following structure:

→4)-β-L-Alt*p*-(1→4)-β-D-Glc*p*-(1→3)-4-*O*-[(*R*)-1-carboxyethyl]-β-D-Glc*p*-(1→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→
2
↑
1
β-D-Glc*p*

Keywords: *Butyrivibrio fibrisolvens*; Bacterial polysaccharide; (1-Carboxyethyl)-galactose; (1-Carboxyethyl)-glucose; L-Altrose

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1. Introduction

Butyrivibrio fibrisolvens is a strictly anaerobic bacterium most commonly isolated from the gastrointestinal tract of ruminant animals. Though principally involved in the catabolism of plant-derived polysaccharides, most strains of *B. fibrisolvens* also produce significant amounts of extracellular polysaccharides when grown in pure culture [1]. Previously, we have reported that 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].

We have also reported the detailed structures of the repeating units for the extracellular polysaccharides produced by *B. fibrisolvens* strains X6C61 [3] and 49 [5]. We continue these studies with other selected strains to define the structures of the repeating units of the major classes of extracellular polysaccharides produced by *B. fibrisolvens*. Previous studies with strain CF3, a bacterial isolate from the ovine ceccum [6], have shown the presence of D-glucose, two unidentified (1-carboxyethyl)hexoses, and the unusual L-altrose, found for the first time in nature in this polysaccharide [2]. 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 NMR spectrum of the polysaccharide displayed broad peaks and contained, inter alia, signals from two methyl groups at δ 1.39 and 1.36 together corresponding to six protons, and from five anomeric protons at δ 5.23, 5.10, 4.80, 4.66, and 4.58. The ^{13}C NMR spectrum showed signals for five anomeric carbons at δ 101.77, 99.56 (two carbons), 104.07, and 103.08, corroborating the presence of a pentasaccharide repeating unit. The ^{13}C NMR spectrum also contained signals for two carbonyl carbons at δ 181.3 and 181.1, and for two methyl carbons at δ 19.3 and 18.4, indicating the presence of the two (1-carboxyethyl)-sugars reported previously [1,2].

The polysaccharide was carboxyl-reduced [7] using $NaBH_4$ as the reducing agent. A ^{1}H NMR spectrum of the product showed that the signals from the two methyl groups, originally at δ 1.39 and 1.36 in the unreduced polysaccharide, were shifted to 1.19 and 1.17, though not in a quantitative fashion. The reduction procedure was therefore repeated. A ^{1}H NMR spectrum of the re-reduced polysaccharide indicated

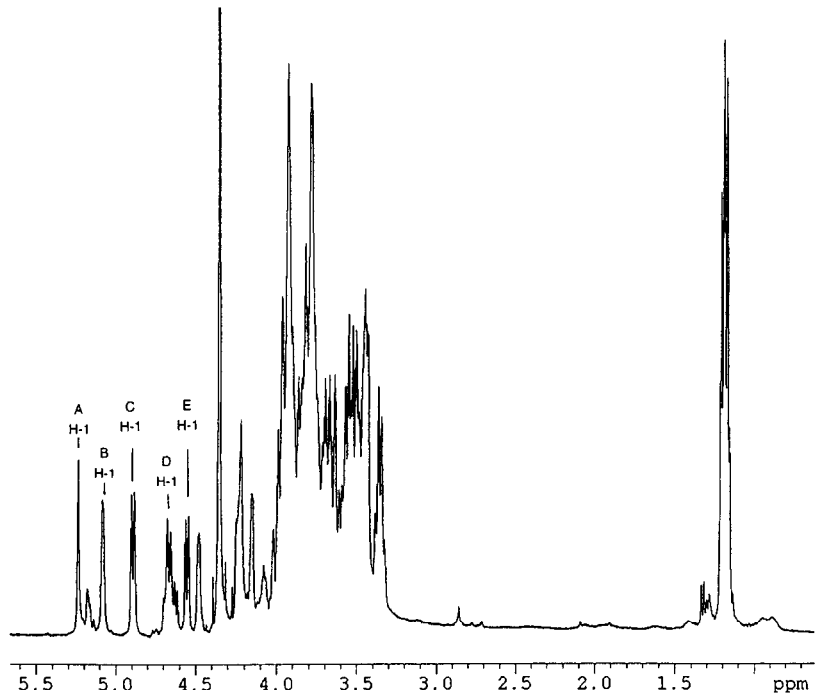


Fig. 1. 400-MHz ^{1}H NMR spectrum of the carboxyl-reduced extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain CF3. For an explanation of **A–E**, see Table 1.

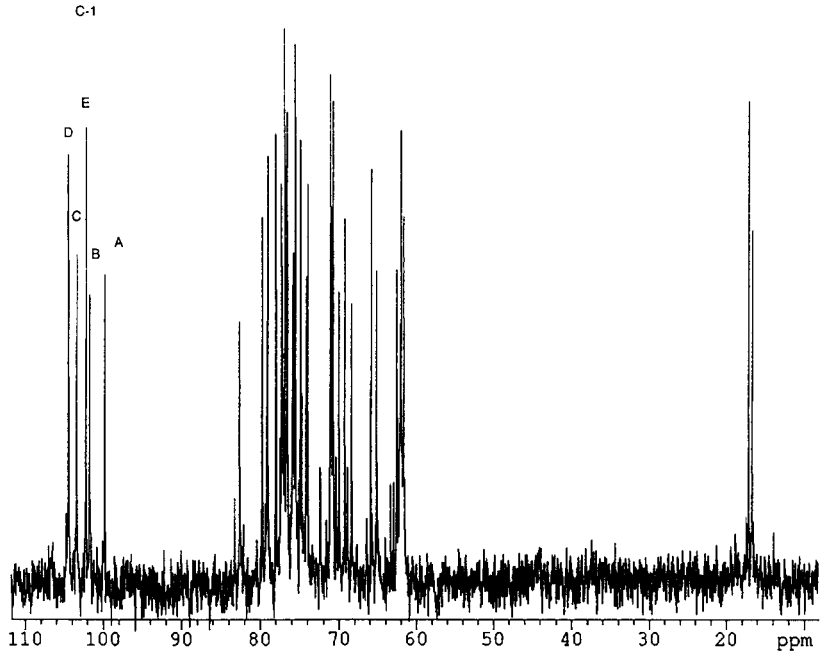


Fig. 2. 100.5-MHz ^{13}C NMR spectrum of the carboxyl-reduced extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain CF3. For an explanation of **A–E**, see Table 1.

that the reduction was now essentially complete. The ^{1}H and ^{13}C NMR spectra (Figs. 1 and 2) and an HMQC spectrum of this product showed signals from five anomeric protons and carbons (Table 1), which corroborated the presence of a pentasaccharide repeating unit. The ^{13}C NMR spectrum also had signals at δ 17.13 and 16.69 corresponding to the two methyl group carbons in the two (2-hydroxy-1-methylethyl)hexoses.

Acid hydrolysis of the native polysaccharide, reduction with $NaBH_4$ and acetylation, gave the acetate of 1,6-anhydroaltrose and the alditol acetates of altrose and glucose in the relative proportions 0.6:0.4:2.5, confirming the ratio of one altrose to two glucose residues in the polysaccharide, as previously reported [2]. The L configuration of the altrose unit was reported earlier [2] and the D configuration of the glucose unit was now determined by GLC of the trimethylsilylated (+)-2-butyl glycosides [8].

The polysaccharide was carboxyl-reduced [7] with $NaBD_4$, and, as in the case above, the procedure needed to be repeated in order to insure a more fully reduced product. The deuterium-reduced polysaccharide was hydrolyzed, the released sugars reduced with

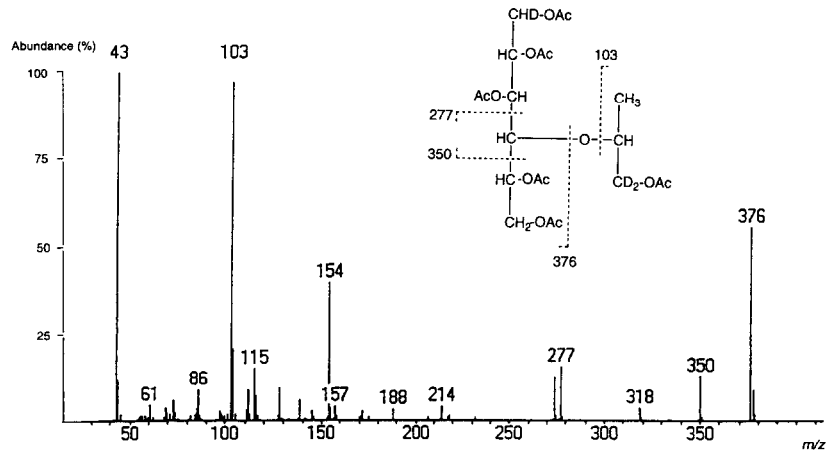


Fig. 3. Mass spectrum and the most abundant fragments for the alditol-1-*d* acetate of 4-*O*-[(*R*)-2-hydroxy-1-methylethyl-2,2-d_2]-D-glucose.

Table 1
^{1}H and ^{13}C NMR data obtained at 70 °C for the carboxyl-reduced polysaccharide from *Butyrivibrio fibrisolvens* strain CF3

Residue	Chemical shifts (δ)									
								2-Hydroxy-1-methylethyl		
	H-1 C-1	($J_{H-1,H-2}$, Hz) ($J_{C-1,H-1}$, Hz)	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a/H-6b C-6	H-1 C-1	H-2a/H-2b C-2	1-Methyl C
→ 2,4)-β-L-Alt*p*-(1 → **(A)**	5.23 99.77	(< 2) (164)	4.15 77.10	4.48 68.31	3.92 75.48	3.90 73.80	3.78/3.96 62.50			
→ 4)-[6-*O*-R]-α-D-Gal*p*-(1 → [a] **(B)**	5.08 101.69	(3.3) (171)	3.92 69.94	3.98 70.88	4.23 79.68	4.24 70.30	3.76/3.81 69.21	3.84 78.97	3.48/3.78 65.10	1.19 17.13
→ 4)-β-D-Glc*p*-(1 → **(C)**	4.89 103.31	(7.6) (166)	3.36 74.79	3.69 76.52	3.62 79.10	3.54 75.70	3.79/4.00 61.92			
→ 3)-[4-*O*-R]-β-D-Glc*p*-(1 → [a] **(D)**	4.66 104.41	(8.4) (161)	3.56 75.69	3.86 82.57	3.42 76.30	3.53 75.48	3.69/3.80 62.09	3.71 77.93	3.54/3.64 65.76	1.17 16.69
β-D-Glc*p*-(1 → **(E)**	4.55 102.14	(7.6) (161)	3.34 73.90	3.50 76.52	3.43 70.69	3.45 77.22	3.77/3.92 61.58			

[a] R = (*R*)-2-hydroxy-1-methylethyl.

Table 2
Observed ^1H and ^{13}C chemical shifts [a] of the α and β forms of the isolated 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose in D_2O at 70 °C

Anomer	Chemical shifts (δ)								
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-1′	H-2′
	C-1	C-2	C-3	C-4	C-5	C-6		C-1′	C-2′
	$\Delta\delta_C$ [b]								
α	5.19	3.53	3.82	3.31	3.84	3.78	3.89	4.05	1.37
	92.08	71.95	72.31	78.72	68.24	60.37		77.44	19.12
	−0.91	−0.52	−1.47	8.01	−4.13	−1.47			
β	4.62	3.24	3.61	3.32	3.47	3.72	3.86	4.05	1.36
	95.79	74.50	74.92	78.78	75.34	60.27		77.44	19.65
	−1.05	−0.70	−1.84	8.07	−1.42	−1.57			

[a] The chemical shifts of the carbonyl carbons were not determined.
[b] ^{13}C chemical shift differences between the observed values and the literature data [12] for α- and β-D-glucose ($\delta_{observed} - \delta_{literature}$).

$NaBD_4$ and acetylated. The products were analyzed by GLC–MS, yielding the acetate of 1,6-anhydroaltrose and the alditol-1-*d* acetates of altrose, glucose, 6-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-galactose, and (2-hydroxy-1-methylethyl-2,2-d_2)-hexose in the ratio 1.2(1,6-anhydroaltrose and altrose):2.1:1.0:0.7, confirming the presence of one altrose, two glucose, one 6-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-galactose, and one (2-hydroxy-1-methylethyl-2,2-d_2)-hexose residue in the carboxyl-reduced polysaccharide.

The alditol acetate of 6-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-galactose was compared on GLC–MS with those prepared from the (*R*)- and (*S*)-forms of carboxyl-reduced synthetic methyl 6-*O*-(1-carboxyethyl)-D-galactopyranoside [9]. The retention times on GLC showed the component to be 6-*O*-[(*R*)-2-hydroxy-1-methylethyl-2,2-d_2]-D-galactose. This is the reduced form of 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose, which consequently is the component in the native polysaccharide. This sugar has also been detected in the extracellular polysaccharides produced by the *B. fibrisolvens* strains X6C61 [3] and 49 [5].

The mass spectrum of the alditol acetate of the second (2-hydroxy-1-methylethyl-2,2-d_2)-hexose indicated a 4-*O*-[2-hydroxy-1-methylethyl-2,2-d_2]-hexose [10]. The spectrum and the origin of the principal fragments are depicted in Fig. 3.

To further investigate the structure of this component, the native polysaccharide was hydrolyzed, and the acidic sugars were first separated from the neutral sugars using an anion-exchange column. The mixture of the two (1-carboxyethyl)hexoses was then further fractionated by chromatography on a cation-exchange column in the Ca^{2+}-form [11]. ^1H NMR analysis of the fractions showed that one fraction contained the unknown pure (1-carboxyethyl)hexose. Treatment of an aliquot from the fraction containing the pure unknown (1-carboxyethyl)hexose with BBr_3, using a modification of the procedure described by Hough and Theobald [12] gave glucose, as determined by sugar analysis. The D configuration of the resulting glucose was established according to Gerwig et al. [8]. These data together with the data from the sugar analysis of the deuterium-reduced polysaccharide indicated the second (1-carboxyethyl)hexose to be 4-*O*-(1-carboxyethyl)-D-glucose. The ^1H and ^{13}C NMR signals of the isolated (1-carboxyethyl)hexose, assigned using different 2D NMR techniques (H,H-COSY, relay and double relay H,H-COSY and HMQC, see Table 2), were also consistent with a structure composed of a glucose unit with a 1-carboxyethyl substituent at O-4. This was evident from the downfield shift of the signal from C-4 (+8.0 and +8.1 ppm for the α and the β form, respectively) and the smaller upfield shift of the signals from the vicinal carbons (Table 2). To determine the absolute configuration of the 1-carboxyethyl substituent, the alditol acetate of 4-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-D-glucose obtained from the carboxyl-reduced polysaccharide was compared on GLC–MS with the alditol acetate of 4-*O*-[(*S*)-2-hydroxy-1-methylethyl]-D-glucose prepared from the carboxyl-reduced [7] extracellular polysaccharide from *Aerococcus viridans* var *homari* [13]. The different retention time of the model compound showed the polysaccharide constituent to be 4-*O*-

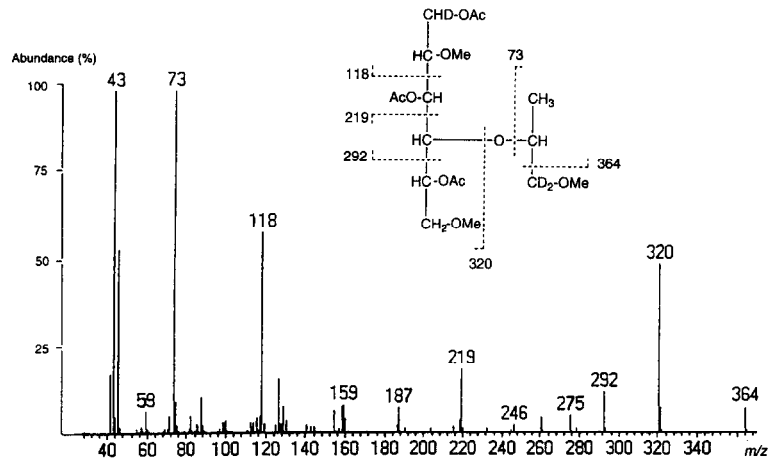


Fig. 4. Mass spectrum and the most abundant fragments for the alditol-1-*d* acetate of 2,6-di-*O*-methyl-4-*O*-[(*R*)-2-methoxy-1-methylethyl]-D-glucose.

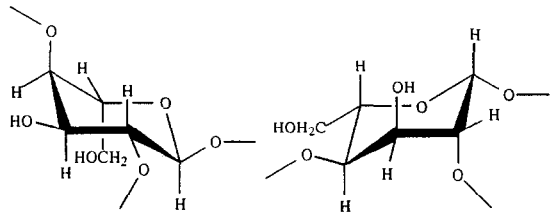
[(*R*)-2-hydroxy-1-methylethyl]-D-glucose. This is the reduced form of 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose, which consequently is the component in the native polysaccharide.

Methylation of the native polysaccharide was performed using sodium methylsulfinyl anion and CH_3I [14]. The resulting methyl esters of the permethylated polysaccharide were reduced with a solution of lithium triethylborodeuteride (Superdeuteride) [15]. The reduced, methylated polysaccharide was hydrolyzed, and the released sugars were reduced with $NaBH_4$ and acetylated. The products were analyzed by GLC–MS, which showed the alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 3,6-di-*O*-methyl-L-altrose, 2,3-di-*O*-methyl-6-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-D-galactose, and 2,6-di-*O*-methyl-4-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-D-glucose. However, the acetate of 2,6-di-*O*-methyl-4-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-D-glucitol gave few diagnostic fragments.

To confirm the above findings, a $NaBH_4$ carboxyl-reduced polysaccharide was methylated and hydrolyzed, and the released sugars were reduced with $NaBD_4$ and acetylated. Analysis of the products by GLC–MS gave the alditol-1-*d* acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 3,6-di-*O*-methyl-L-altrose, 2,3-di-*O*-methyl-6-*O*-(2-methoxy-1-methylethyl)-D-galactose, and 2,6-di-*O*-methyl-4-*O*-(2-methoxy-1-methylethyl)-D-glucose. Fig. 4 shows the 70 eV EIMS spectrum of the acetate of 2,6-di-*O*-methyl-4-*O*-(2-methoxy-1-methylethyl)-D-glucitol-1-*d* together with the origin of the most abundant fragments. The above data indicated the repeating unit of the polysaccharide to be composed of a terminal D-glucose, a 4-substituted D-glucose, a 2,4-disubstituted L-altrose, a 4-substituted 6-*O*-(1-carboxyethyl)-D-galactose, and a 3-substituted 4-*O*-(1-carboxyethyl)-D-glucose residue.

Information on the respective sugar residues and the anomeric configurations could be deduced from the ^1H and ^{13}C NMR data of the carboxyl-reduced polysaccharide (Table 1). Using different 1D and 2D experiments, H,H-COSY, relay and double relay H,H-COSY, the spin-systems attributable to each anomeric proton could be assigned and the $J_{1,2}$ values determined. HMQC experiments allowed then the assignment of the corresponding carbon signals and the $^1J_{C,H}$ values. The substitution positions of the residues were revealed by the high chemical shifts of the substituted carbon signals. From the pattern of several cross-peaks in the phase-sensitive COSY spectrum, the size of the coupling constants from the coupling between ring protons could be estimated. This information together with published chemical shift data for the sugars [3,5,16,17] allowed the assignment of the different spin-systems to specific sugar residues. The NMR data also showed that the 4-substituted 6-*O*-(1-carboxyethyl)-D-galactose was an α-pyranoside whereas the two D-glucose and the 3-substituted 4-*O*-(1-carboxyethyl)-D-glucose residues were β-pyranosides. The determination of the anomeric configuration of L-altrose required some additional considerations. Free altrose in solution is in equilibrium between the $^1C_4$ and $^4C_1$ chair conformations [18], and as the former would have an axial hydroxyl substituent in the 2-position, the $^3J_{H\text{-}1,H\text{-}2}$ value is not a reliable indicator of the anomeric configuration. The observed $^1J_{C,H}$ value (164 Hz) indicates that H-1 of L-altrose in the carboxyl-reduced polysaccharide is in an axial position [19]. This

fact indicates that the L-altrose residue must be in either the α configuration in a $^4C_1$ conformation (**1**) or in the β configuration in a $^1C_4$ conformation (**2**).



1 2

The small $^3J_{H\text{-}1,H\text{-}2}$ value found (< 2 Hz) precludes the first possibility (**1**), since in that case H-1 is in a trans-diaxial relationship with H-2, and the expected value should be 7–8 Hz. The measured $^3J_{H\text{-}1,H\text{-}2}$ value is instead in good agreement with the value expected for an axial-equatorial relationship, as shown in **2**. The above findings were corroborated by the ^3J values between vicinal ring protons in the L-altrose residue (< 2, 4.3, and < 2 Hz, for $^3J_{H\text{-}1,H\text{-}2}$, $^3J_{H\text{-}2,H\text{-}3}$, and $^3J_{H\text{-}3,H\text{-}4}$, respectively) measured from a resolution-enhanced ^{1}H NMR spectrum, the cross-peak patterns from a phase-sensitive H,H-COSY spectrum and from NOE difference experiments irradiating the H-1, H-2, and H-3 signals. The NOE connectivity between H-1 (δ 5.23) and H-5 (δ 3.35) observed in a NOESY experiment indicated a 1,3-diaxial relationship for these protons, and thus the L-altrose residue in the reduced polysaccharide preferentially adopts the $^1C_4$ conformation (**2**).

In order to deduce the sequence of the sugar residues, the carboxyl-reduced polysaccharide was subjected to a Smith degradation [20]. It was first oxidized with sodium periodate, then reduced with sodium borohydride, and the resulting polymer was isolated by dialysis. A ^{1}H NMR spectrum of the product indicated incomplete oxidation, thus the procedure was repeated. Sugar analysis of the final product gave L-altrose, 1,6-anhydro-L-altrose, 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-glucose, erythritol, and 1-*O*-(2-hydroxy-1-methylethyl)-threitol identified by GLC–MS as their alditol acetates. The identity of 1-*O*-(2-hydroxy-1-methylethyl)-threitol was confirmed by comparison with an authentic sample prepared by Smith degradation of the reduced *B. fibrisolvens* strain 49 capsular polysaccharide [5]. The ^{1}H NMR spectrum of the polymer showed only two anomeric proton signals (indicating two intact sugar residues), in agreement with the sugar analysis. From the chemical shifts and coupling constants (δ 5.19, $J_{1,2} < 2$ Hz and δ 4.64, $J_{1,2}$ 7.6 Hz) of the signals, they could be assigned to the 2,4-disubstituted β-L-altrose residue and the 3-substituted 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-β-D-glucose residue, respectively.

Mild acid hydrolysis (0.5 M trifluoroacetic acid at room temperature) of the polymer mentioned above followed by separation of the hydrolysis products by gel filtration on a column of Bio-Gel P-2 yielded two saccharides. Sugar analysis of the first eluting saccharide gave equimolar amounts of 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-glucose and 1-*O*-[2-hydroxy-1-methylethyl]-D-threitol. The ^{1}H NMR spectrum showed one anomeric proton signal at δ 4.59 ($J_{1,2}$ 7.9 Hz), confirming the β configuration of the 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-glucose residue. The above data indicated the presence of the disaccharide sequence **3** in the carboxyl-reduced polysaccharide.

4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-β-D-Glc*p*-(1 → 4)-6-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-Gal*p* **3**

Sugar analysis of the second saccharide gave L-altrose + 1,6-anhydro-L-altrose and erythritol in equimolar amounts. The ^{1}H NMR spectrum showed only one anomeric proton signal at δ 5.05 ($J_{1,2} < 2$ Hz), corresponding to the β-L-altrose residue. These data indicated the presence of a second disaccharide sequence (**4**) in the carboxyl-reduced polysaccharide.

β-L-Alt*p*-(1 → 4)-D-Glc*p* **4**

Additional sequence information on the carboxyl-reduced polysaccharide was obtained by inter-residue NOEs between the anomeric proton of one residue and the proton on the linkage carbon of the next residue. The inter-residue NOEs obtained for all disaccharide elements in the repeating unit of the polysaccharide are shown in Table 3. A NOE connectivity was observed between H-1 of the 4-substituted D-glucose residue and H-3 of the 3-substituted 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-glucose residue, and between H-1 of the 4-substituted 6-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-galactose residue and H-4 of the 2,4-disubstituted L-altrose residue. These data indicated that the compounds **3** and **4**, obtained from the Smith degradation, originated from the backbone structure of the carboxyl-reduced polysaccharide, and showed the backbone structure of the carboxyl-reduced polysaccharide to be the following (**5**):

→ 3)-4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-β-D-Glc*p*-(1 → 4)-6-*O*-[(*R*)-2-hydroxy-1-methylethyl]-α-D-Gal*p*-(1 → 4)-β-L-Alt*p*-(1 → 4)-β-D-Glc*p*-(1 → **5**

→4)-β-L-Alt*p*-(1→4)-β-D-Glc*p*-(1→3)-4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-β-D-Glc*p*-(1→4)-6-*O*-[(*R*)-2-hydroxy-1-methylethyl]- α-D-Gal*p*-(1→
2
↑
1
β-D-Glc*p*

6

The linkage of the terminal β-D-glucose unit to O-2 of the L-altrose residue was confirmed by a NOE cross-peak between its H-1 atom and H-2 of the latter.

The NOE connectivity between H-1 of the 4-substituted glucose residue and H-2 of the 3-substituted 4-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-glucose residue and the relative high chemical shift value for these protons indicated a conformation of the (1 → 3) glycosidic bond with a short distance between these protons. This conformation could be due to steric interactions between the 4-substituted glucose at O-3 and the 2-hydroxy-1-methylethyl substituent at O-4.

A NOE connectivity between the methyl group at δ 1.19 and H-6 of 6-*O*-[(*R*)-2-hydroxy-1-methylethyl]-D-galactose allowed the assignment of the 2-hydroxy-1-methylethyl groups.

According to all of the above data, the structure of the carboxyl-reduced polysaccharide must be the one above (**6**).

The above sequence was further confirmed by the observed inter-residual three-bond $^{13}C-^{1}H$ couplings between the anomeric proton of one residue and the substituted carbon of the next, as detected in an HMBC experiment. The cross-peaks of the anomeric protons were examined, and both intra- and inter-residual connectivities were found. Cross-peaks were observed between H-1 of L-altrose (δ 5.23) and C-4 of 4-substituted D-glucose (δ 79.10), H-1 of the latter residue (δ 4.89) and C-3 of 3-substituted 4-*O*-(2-hydroxy-1-methylethyl)-D-glucose (δ 82.57), H-1 of the latter residue (δ 4.66) and C-4 of 6-*O*-(2-hydroxy-1-methylethyl)-D-galactose (δ 79.68), and between H-1 of terminal D-glucose (δ 4.55) and C-2 of L-altrose (δ 77.10). No peak from inter-residual couplings was detected from the 6-*O*-(2-hydroxy-1-methylethyl)-D-galactose residue. The results were further supported by the observed $^{3}J_{C,H}$ connectivities between the anomeric carbon of one residue and the proton on the substituted carbon of the next, detected in an HMBC experiment (Table 4).

Thus the structure of the native *B. fibrisolvens* strain CF3 extracellular polysaccharide must be the one below.

Although the structure of the CF3 polysaccharide shown above is quite different from the structures, we have reported previously for strains 49 [5] and X6C61 [3], there are in fact several commonalities more apparent upon closer examination. The polysaccharides of all three strains contain 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose, which to date has been found only in *B. fibrisolvens*. In all three strains this unusual acidic sugar is in the α-pyranosyl configuration, and is linked in turn to the 4-position of a hexose. In strains 49 and X6C61 this hexose is an α-D-Galp residue, while in strain CF3 it is a β-L-Alt*p* unit. While superficially these two sugars appear to be quite different, in fact L-Alt is the C-5 epimer of D-Gal. Inversion of configuration would accompany enzymatic epimerization, leading to the formation of a β-L-Alt*p* residue from an α-D-Gal*p* precursor molecule in an analogous way that has already been described for enzymes involved in bacterial alginate [21] and mammalian heparan sulfate [22] biosyntheses. In the former, β-D-ManA is enzymatically converted to α-L-GulA, while in the latter β-D-GlcA is converted to α-L-IdoA. *B. fibrisolvens* strain CF3 may contain a unique C-5 epimerase with specificity for α-D-Gal*p* residues, and we have initiated studies to test this hypothesis. Interestingly, we have also previously inferred [6] the existence of what is probably a different C-5 epimerase in *B. fibrisolvens* strain X6C61 responsible for L-iduronate biosynthesis, though the specificities of neither enzyme nor their degree of relatedness is as yet known.

In all three strains studied to date, the hexose linked to 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose — α-D-Gal*p* or β-L-Alt*p* — is in turn further substi-

→4)-β-L-Alt*p*-(1→4)-β-D-Glc*p*-(1→3)-4-*O*-[(*R*)-1-carboxyethyl]-β-D-Glc*p*-(1→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→
2
↑
1
β-D-Glc*p*

Table 3
Observed NOEs in the NOESY spectrum for anomeric protons in the carboxyl-reduced polysaccharide from *Butyrivibrio fibrisolvens* strain CF3

H-1 δ; residue	NOE δ; residue, proton
5.23; → 2,4)-β-L-Alt*p*-(1 → **(A)**	4.15; **A**, H-2 3.62; **C**, H-4 3.35; **A**, H-5
5.08; → 4)-[6-*O*-R]-α-D-Gal*p*-(1 → [a] **(B)**	3.92; **A**, H-4 [b] 3.92; **B**, H-2
4.89; → 4)-β-D-Glc*p*-(1 → **(C)**	3.86; **D**, H-3 3.69; **C**, H-3 3.56; **D**, H-2
4.66; → 3)-[4-*O*-R]-β-D-Glc*p*-(1 → [a] **(D)**	4.23; **B**, H-4 3.86; **D**, H-3 3.53; **D**, H-5
4.55; β-D-Glc*p*-(1 → **(E)**	4.15; **A**, H-2 3.50; **E**, H-3 3.45; **E**, H-5

[a] R = (*R*)-2-hydroxy-1-methylethyl.
[b] Coincides with the intra-residual NOE of H-2.

tuted in a strain-specific way. Thus an α-L-Rha*p* substitution is found at O-2 of α-D-Gal*p* in strain X6C61, an *O*-acetyl group is located at O-3 of α-D-Gal*p* in strain 49, and a β-D-Glc*p* residue is attached to O-2 of β-L-Alt*p* in strain CF3. This creates wide divergencies in the resulting structures, even though such structures may have evolved from a common precursor epitope. Lending some additional support for this concept is the observation that in all three strains studied to date, the α-D-Gal*p* or β-L-Alt*p* residue is in turn linked to O-4 of a β-D-Glc*p* residue, which is the next sugar in the polysaccharide chain.

The polysaccharide of *B. fibrisolvens* strain CF3 also contains 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose, another rather unusual monosaccharide which has been previously identified in the capsular polysaccharide of *Klebsiella* serotype 66 [10] and in the type 3 O-antigen of *Shigella dysenteria* [23]. The corresponding diastereomer of this sugar has been identified in the capsular polysaccharide of *Aerococcus viridans* var *homari* [13]. This sugar has not been found in the polysaccharides of either *B. fibrisolvens* strain 49 or X6C61, but is apparently present in related strains isolated by Lewis and Dehority [24] as we have previously reported [1].

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 240 °C at 3 °C/min. GLC–MS was performed on a Hewlett–

Table 4
Observed $^2J_{C,H}$ and $^3J_{C,H}$ connectivities from anomeric carbons and protons, as observed in a ^{1}H-detected HMBC experiment of the carboxyl-reduced polysaccharide from *Butyrivibrio fibrisolvens* strain CF3

$J_{C,H}$ connectivities to ^{1}H atom δ; residue, hydrogen	Anomeric carbon (δ, ppm)	Residue	Anomeric proton (δ, ppm)	$J_{C,H}$ connectivities to ^{13}C atom δ; residue, carbon
4.48; **A**, H-3 3.62; **C**, H-4	99.77	→ 2,4)-β-L-Alt*p*-(1 → **(A)**	5.23	79.10; **C**, C-4 77.10; **A**, C-2
–	101.69	→ 4)-[6-*O*-R]-α-D-Gal*p*-(1 → [a] **(B)**	5.08	70.88; **B**, C-3
3.86; **D**, H-3 3.36; **C**, H-2	103.31	→ 4)-β-D-Glc*p*-(1 → **(C)**	4.89	82.57; **D**, C-3
4.23; **B**, H-4 3.56; **D**, H-2	104.41	→ 3)-[4-*O*-R]-β-D-Glc*p*-(1 → [a] **(D)**	4.66	79.68; **B**, C-4
4.15; **A**, H-2 3.34; **E**, H-2	102.14	β-D-Glc*p*-(1 → **(E)**	4.55	77.10; **A**, C-2

[a] R = (*R*)-2-hydroxy-1-methylethyl.

Packard 5970 MSD instrument, using the column and conditions mentioned above. Methylation analysis was performed as described [25] and the methylated products were recovered using a Sep-Pak C-18 cartridge [26]. NMR spectra were recorded for solns in D_2O at 70 °C, using a Varian VXR 400 instrument. The native polysaccharide samples were passed through a column of Dowex 50 (Na^+-form) prior to NMR analysis. ^1H and ^{13}C chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropionate-d_4 (TSP, δ_H 0.00) and acetone (δ_C 31.07), respectively, as internal references. 2D (COSY, relay and double relay COSY, NOESY, HMBC, and HMQC) experiments were performed according to standard pulse sequences available in the Varian software. A 90° pulse was used in the correlation experiments, mixing times of 0.3 and 0.6 s in the NOESY experiments, and a delay time of 60 ms in the HMBC experiments.

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.

Organism, growth conditions, and isolation of extracellular polysaccharides.—*B. fibrisolvens* strain CF3, originally isolated by Lewis and Dehority [24], was grown on 1% glucose at 39 °C under anaerobic conditions on the defined medium of Cotta and Hespell [27] supplemented with 0.3% Trypticase. Cells were removed from stationary-phase cultures by centrifugation, and crude extracellular polysaccharide was phenol-extracted, dialyzed, and lyophilized as previously described [2]. The crude polysaccharide was further purified by anion-exchange chromatography. In a typical run, crude polysaccharide (200 mg) dissolved in H_2O (200 mL) was applied to a column (1.5 × 12 cm) of DEAE-Sepharose 6-B. The column was eluted first with water (200 mL) and then with a linear gradient of aq NaCl (0–1 M, 260 mL). Aliquots (0.2 mL) of the fractions were analyzed for carbohydrate using the phenol/H_2SO_4 procedure, and the tubes containing the polysaccharide were pooled, dialyzed against H_2O, and recovered after freeze-drying. Typically, this procedure yielded 80–90 mg of purified acidic polysaccharide.

Isolation of the unknown (1-carboxyethyl)hexose.—Polysaccharide (20 mg) was hydrolyzed with 2 M CF_3CO_2H (2 mL) for 1 h at 120 °C; the hydrolysate was freeze-dried, redissolved in H_2O (1 mL), and the soln was applied to a small column (1 × 2 cm) of Dowex 1X8-50 (OH^--form). After elution of the neutral sugars with H_2O (5 mL), the acidic sugars were eluted with 1 M NH_4OH (5 mL). The ammonia soln was freeze-dried, redissolved in H_2O (1 mL), and the soln was applied to a column (1.6 × 70 cm) of Dowex 50W-X8 (200–400 mesh; Ca^{2+}-form). The column was irrigated with H_2O, and the eluent was monitored with a differential refractometer. The fractions were analyzed by ^1H NMR spectroscopy. Fraction 1 contained only the unknown (1-carboxyethyl)hexose (1.5 mg). Part of this product was de-alkylated with BBr_3 using a modification of the procedure described by Hough and Theobald [12]. (1-Carboxyethyl)hexose (0.2 mg) was suspended under N_2 in CH_2Cl_2 (0.75 mL), cooled to −70 °C, and 16% (v/v) BBr_3 in CH_2Cl_2 (0.25 mL, −70 °C) prepared under N_2, was added. The mixture was allowed to react in a Teflon-capped sealed tube for 30 min at −70 °C, and then permitted to gradually regain room temperature over a period of 10 h. The solvent and excess reagents were evaporated under a stream of N_2, the residue redissolved in MeOH (0.3 mL), and then dried with a stream of N_2. The washed, dried residue was treated with 10% (v/v) CF_3CO_2H (0.25 mL, 100 °C, 1 h), to afford a hexose that was identified by GLC and GLC–MS of its alditol acetate derivative. Its absolute configuration was determined according to Gerwig et al. [8].

Carboxyl reduction of the native polysaccharide.—Reduction was performed according to Taylor et al. [7] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and either $NaBH_4$ or $NaBD_4$ as the reducing agent. The procedure was repeated twice in order to obtain a more complete reduction. The reaction products were checked by ^1H NMR spectroscopy to monitor the reduction efficiency.

Determination of the configuration of 4-O-[(R)-2-hydroxy-1-methylethyl]-D-glucose.—The acetate of 4-*O*-(2-hydroxy-1-methylethyl-2,2-d_2)-D-glucose, obtained from the carboxyl-reduced ($NaBD_4$) polysaccharide [7], was compared by GLC–MS with the acetate of 4-*O*-[(*S*)-2-hydroxy-1-methylethyl]-D-glucose prepared from the carboxyl-reduced [7] extracellular polysaccharide from *Aerococcus viridans* var *homari* [13] using the same conditions as in the sugar analysis.

Smith degradation.—Carboxyl-reduced ($NaBH_4$) polysaccharide (27 mg) was treated with a soln of $NaIO_4$ (67 mg) in NaOAc buffer (0.1 M, pH 3.7; 2.5 mL) for 20 h at 4 °C. Excess $NaIO_4$ was destroyed by addition of ethylene glycol (50 μL), and the product was reduced with $NaBH_4$ (30 mg in 3.0 mL 1 M NH_4OH), dialyzed against distilled water, and then

freeze-dried. A ^{1}H NMR spectrum of the product indicated incomplete oxidation, so the above procedure was repeated to obtain a more completely oxidized product (21 mg). The polymer was treated with 0.5 M CF_3CO_2H (0.5 mL) at room temperature for 24 h, then the soln was freeze-dried, and the products were separated by gel filtration on a column (1.5 × 70 cm) of Bio-Gel P-2. The fractions were analyzed by ^{1}H NMR spectroscopy. A portion of each saccharide (≈ 0.3 mg) was hydrolyzed in 2 M CF_3CO_2H for 1 h at 120 °C, the released sugars were reduced with $NaBH_4$ and acetylated with 1:1 Ac_2O–pyridine (0.5 mL) for 1 h at 120 °C. The resulting alditol acetates were analyzed by GLC–MS.

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