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The structure of the exopolysaccharide produced by *Lactobacillus helveticus* 766

Gerard W. Robijn [a], Jerry R. Thomas [a], Han Haas [b], Dick J.C. van den Berg [b], Johannis P. Kamerling [a,*], Johannes F.G. Vliegenthart [a]

[a] *Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands*

[b] *Unilever Research Laboratory Vlaardingen, P.O. Box 114, NL-3130 AC Vlaardingen, The Netherlands*

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Abstract

The exopolysaccharide produced by *Lactobacillus helveticus* 766 in skimmed milk was found to be composed of D-glucose and D-galactose in a molar ratio of 2:1. Linkage analysis and 1D/2D NMR studies (^{1}H and ^{13}C) performed on the native polysaccharide, and on oligosaccharides obtained from a partial acid hydrolysate, showed the polysaccharide to consist of hexasaccharide repeating units with the following structure:

$$\begin{array}{c} \beta\text{-D-Gal}f \\ 1 \\ \downarrow \\ 3 \\ \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow \end{array}$$

Keywords: Lactic acid bacteria; *Lactobacillus helveticus*; Exopolysaccharide structure

1. Introduction

In the food industry, bacterial exopolysaccharides (EPSs) are extensively used as thickening, gelling, and stabilizing agents [1]. Recently, a growing interest has developed in polysaccharides from lactic acid bacteria. Because they are produced by Food

* Corresponding author.

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Grade microorganisms, these polysaccharides may form a new generation of food thickeners. In order to gain a better insight into the relationship between the structures of polysaccharides from lactic acid bacteria and their rheological properties, knowledge of their primary structures is a prerequisite.

In this context, structural studies have so far been carried out on EPSs produced by *Streptococcus thermophilus* [2], *Lactococcus lactis* subsp. *cremoris* SBT 0495 [3], *Lactococcus lactis* subsp. *cremoris* H414 [4], *Lactobacillus delbrückii* subsp. *bulgaricus* rr [5], *Lactobacillus helveticus* TY1-2 [6], and *Lactobacillus sake* 0-1 [7]. Here we report the structure determination of the exopolysaccharide produced by *Lactobacillus helveticus* 766 in skimmed milk.

2. Experimental

Production, isolation, and purification of the exopolysaccharide.—*Lactobacillus helveticus* 766 was grown in skimmed milk for 2 days at 30°C (non-shaken, non-aerated); trichloroacetic acid was then added (final concentration 4% w/v), and the culture was stirred for 2 h. Cells and precipitated proteins were removed by centrifugation (27 000 *g*, 30 min, 4°C). The supernatant was collected and the EPS was precipitated with two volumes of cold EtOH. An aqueous solution of the precipitated material was extensively dialysed against twice-distilled water. After removal of insoluble material by centrifugation, two volumes of EtOH were again added. The resulting precipitate was dissolved in water and subsequently subjected to a fractional precipitation at 30%, 40%, 50%, 60% and 70% acetone, with intermediate centrifugation. The precipitated material collected from the 40% acetone fraction was further purified on a Sephacryl S-500 gel filtration column (150 × 2.2 cm, Pharmacia), eluted with 50 mM NH_4HCO_3, using refractive index monitoring (Bischoff 8100 RI detector). A small amount of the purified polysaccharide (250 μg) was fractionated on a Superose-6 gel filtration column (30 × 1 cm, Pharmacia), eluting with 50 mM NH_4HCO_3, using a Pharmacia FPLC system with refractive index and UV monitoring (Pharmacia, single path monitor UV-1/280 nm).

Protein assay.—Protein contents were determined with the Pierce Protein Assay Reagent, using bovine serum albumin as a standard.

Gas–liquid chromatography and mass spectrometry.—GLC analyses were performed with a Varian 3700 gas chromatograph (temperature program, 130–220°C at 4°C/min), using an SE-30 fused-silica capillary column (25 m × 0.32 mm, Pierce) or a CPSil43 WCOT fused-silica capillary column (25 m × 0.32 mm, Chrompack). Data were collected and processed with a Shimadzu integrator.

Partially methylated alditol acetates were analyzed by GLC–MS with an MD800/8060 system (Fisons instruments; electron energy, 70 eV) equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J&W Scientific). Samples were injected using a split injector (split flow 1/10), and a temperature program of 140–220°C at 4°C/min was used.

Positive-ion mode FAB mass spectra were recorded using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (accelerating voltage, 6 kV; Xe beam). The

methylated oligosaccharide samples were measured over a mass range of m/z 10–1500 in a matrix of *m*-nitrobenzyl alcohol.

Monosaccharide analysis.—Oligo/polysaccharides were subjected to methanolysis (methanolic 1 M HCl, 18 h, 85°C), and the resulting mixtures of methyl glycosides were trimethylsilylated (1:1:5 hexamethyldisilazane–chlorotrimethylsilane–pyridine), and then quantitatively analyzed by GLC on SE-30 [8,9]. In addition, the absolute configurations of the monosaccharides were determined by GLC analysis of the trimethylsilylated (−)-2-butyl glycosides [10] on SE-30.

Methylation analysis.—Samples (native EPS, partially hydrolysed EPS, or oligosaccharide-alditols) were permethylated as described previously [11]. After hydrolysis with 2 M CF_3CO_2H (2 h, 120°C), the partially methylated monosaccharides were reduced with $NaBD_4$. Work-up, comprising neutralization and removal of boric acid by co-evaporation with MeOH, followed by acetylation with Ac_2O (3 h, 120°C) yielded mixtures of partially methylated alditol acetates, which were analysed by GLC on CPSil43 and GLC–MS on DB-1 [8,12].

Partial acid hydrolysis.—Polysaccharide (50 mg) was hydrolysed in 0.3 M CF_3CO_2H (50 mL) for 2 h at 100°C. Then, the solution was lyophilized, and the residue fractionated on a Bio-Gel P-2 (200–400 mesh) gel filtration column (90 × 1.5 cm), eluted with twice-distilled water, using refractive index monitoring. Subfractionation of Bio-Gel P-2 fractions was performed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex LC system, equipped with a CarboPac PA-1 pellicular anion-exchange column (25 cm × 9 mm). The column was eluted with a gradient of NaOAc in 0.1 M NaOH (20–250 mM NaOAc at 5 mM/min) at a flow rate of 4 mL/min. PAD-detection was carried out with a gold working electrode and triple-pulse amperometry (pulse potentials and durations: E_1 0.05 V, 480 ms; E_2 0.60 V, 120 ms; E_3 −0.60 V, 60 ms) was used. Data were collected and processed with a Shimadzu C-R3A integrator. Immediately after collection, the fractions were neutralized with 2 M AcOH, followed by desalting on a cation-exchange resin (Dowex AG 50W-X12, 100–200 mesh, H^+-form, Bio-Rad), and lyophilization. All HPAEC-fractions were reduced with $NaBD_4$ in 1 M NH_4OH prior to analysis. In the cases of oligosaccharide-alditol mixtures, a further fractionation was carried out on CarboPac PA-1 using the same conditions as described above.

NMR spectroscopy.—Proton-decoupled 75.469-MHz ^{13}C NMR spectra were recorded for solutions in D_2O on a Bruker AC-300 spectrometer (Department of Organic Chemistry), equipped with a 5-mm broad-band probe, at a probe temperature of 27°C. Chemical shifts are expressed in ppm downfield from the signal for external tetramethylsilane, but were actually measured by reference to external MeOH (δ 49.00). The data were collected in 16K complex data sets and zero-filled to 64K. After Fourier transformation, using an exponential multiplication, the spectra were baseline corrected with a fourth-order polynomial function.

1D/2D NMR spectra were recorded on a Bruker AMX-500 or a Bruker AMX-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 27°C. Prior to analysis, samples were exchanged twice in D_2O (99.9 atom% D, Isotec) with intermediate lyophilization, and then dissolved in 99.96 atom% D_2O (Isotec). Chemical shifts are expressed in ppm by reference to internal acetone (δ

2.225). Resolution-enhanced 1D ^{1}H NMR spectra were recorded with a spectral width of 5000 Hz (at 500 MHz) in 16K complex data sets. Suppression of the HOD signal was achieved by applying the WEFT pulse sequence as described [13]. The spectra were baseline corrected with a fourth-order polynomial fit when necessary.

2D Homonuclear Hartmann–Hahn (HOHAHA) spectra were recorded using MLEV mixing sequences of 100 ms. The spin-lock field strength corresponded to a 90° pulse width of ca. 27 μs. The spectral widths were between 2008 and 2808 Hz at 500 MHz, or 4800 Hz at 600 MHz in each dimension. The HOD signal was presaturated for 1 s during the relaxation delay. Between 322 and 512 spectra of 2K data points with 2–64 scans per t_1 increment were recorded.

2D Nuclear Overhauser enhancement spectroscopy (NOESY) was performed with a mixing time of 200 ms. The spectral width was 2250 Hz in each dimension. The HOD signal was presaturated for 1 s during the relaxation delay. A total of 512 spectra of 2K data points with 24 scans per t_1 increment were recorded.

2D Rotating-frame nuclear Overhauser enhancement spectra (ROESY) were recorded with mixing times of 225–250 ms. The spectral widths were 5500 Hz at 600 MHz or 2512–3401 Hz at 500 MHz in each dimension. The spin-lock field strength corresponded to a 90° pulse width of ca. 110 μs. The HOD signal was presaturated for 1 s during the relaxation delay. Between 424 and 512 spectra of 2K data points with 8–96 scans per t_1 increment were collected.

2D Double-quantum filtered correlation experiments (COSY) were recorded in 448–512 data sets of 2K data points; 4–34 scans per t_1 increment were collected. The HOD signal was suppressed by presaturation for 1 s during the relaxation delay. The spectral widths were 2008–2808 Hz in each dimension (at 500 MHz).

A phase-sensitive ^{13}C–^{1}H 2D heteronuclear multiple quantum coherence (HMQC) experiment with inverse detection [14] was carried out at a ^{1}H frequency of 500.139 MHz (125.769 MHz for ^{13}C) with a spectral width of 3012 Hz for t_2 and 14000 Hz for t_1, using a 5-mm broad-band probe. The HOD signal was presaturated for 1 s and the signals of the ^{12}C-bound protons were suppressed using a TANGO pulse sequence. ^{13}C Decoupling was not applied during the acquisition of the ^{1}H FID. A total of 512 spectra of 4K data points with 64 scans per t_1 increment were recorded.

All 2D NMR data were processed on Silicon Graphics IRIS workstations (Indigo, Indigo 2 or 4D/35), using TRITON software (Bijvoet Center, Department of NMR Spectroscopy). The time domain data sets were multiplied with a phase-shifted sine bell and, after Fourier transformation and zero-filling, data sets of 1024×2048 points (2048×2048 points for the HMQC experiment) were obtained, which were baseline corrected with a fourth-order polynomial function when necessary.

3. Results and discussion

Isolation, purification, and composition of the polysaccharide.—The crude exopolysaccharide (EPS) produced by *Lactobacillus helveticus* 766, obtained as an ethanol precipitate from the TCA-treated medium, was purified by fractionated acetone precipitation and subsequent gel filtration chromatography on Sephacryl S-500. The protein

Table 1
Methylation analysis data of native EPS (**1**), and native EPS after mild acid hydrolysis (**2**)

Derivative	Molar amounts [a]	
	1	**2**
2,3,5,6-Gal [b]	0.9	0.5
2,3,4,6-Gal	–	0.3
2,3,4,6-Glc	–	–
2,4,6-Glc	1.0	1.0
2,3,6-Glc	–	0.7
2,3,4-Glc	1.8	1.8
2,3,4-Gal	0.9	0.9
2,6-Glc	1.1	0.4

[a] 2,4,6-Glc is taken as 1.0.
[b] 1,4-Di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-D-galactitol-*1-d*, etc.

content of the purified material was less than 1%. Additional analysis of the material on Superose-6 afforded the polysaccharide as a sharp void-volume peak, suggesting a polymer of high molecular mass.

Monosaccharide analysis of native EPS (**1**), including determination of absolute configurations, revealed a composition of D-Glc and D-Gal in a molar ratio of 2.0:1.0. Methylation analysis of **1** (Table 1) showed the presence of terminal galactofuranose, 3-substituted glucopyranose, 6-substituted glucopyranose, 6-substituted galactopyranose, and 3,4-disubstituted glucopyranose (or 3,5-disubstituted glucofuranose) in molar ratios of 1:1:2:1:1. According to NMR experiments (vide infra) the disubstituted glucosyl residue is in the pyranose ring form. To obtain information about the position of the acid-labile terminal galactofuranosyl group [15], a methylation analysis was also carried out on EPS after mild acid hydrolysis (0.3 M CF_3CO_2H, 7 min, 100°C; **2**), revealing a partial conversion of 3,4-disubstituted glucose into 4-substituted glucose (Table 1). This finding demonstrates that in the native polysaccharide the terminal galactofuranosyl group is attached to O-3 of the 3,4-disubstituted glucose residue. The terminal galactopyranose derivative observed in the methylation analysis of **2** originates from hydrolytically released galactose.

The 1D ^{1}H NMR spectrum of **1** (Fig. 1) contains six signals in the anomeric region (δ 5.6–4.4), corresponding with a hexasaccharide repeating unit. The six monosaccharide units were arbitrarily labelled **A**–**F**. Based on its downfield chemical shift and its small coupling constant, the anomeric signal at δ 5.488 (residue **F**, $^3J_{1,2} < 2$ Hz) was assigned to H-1 of the galactofuranosyl group. The coupling constants of the anomeric signals at δ 4.528 (residue **A**, $^3J_{1,2}$ 7.7 Hz) and δ 4.537 (residue **B**, $^3J_{1,2}$ 7.8 Hz) suggest the presence of two β-hexopyranosyl residues, whereas the coupling constants of the resonances at δ 4.963 (residue **C**, $^3J_{1,2}$ 3.7 Hz), δ 4.991 (residue **D**, $^3J_{1,2}$ 3.7 Hz), and δ 5.295 (residue **E**, $^3J_{1,2}$ 3.8 Hz) indicate three α-hexopyranosyl residues.

The 1D ^{13}C NMR spectrum of **1** (not shown) is in agreement with the suggested hexasaccharide repeating unit, since six signals are observed in the anomeric region (δ 110–95). Based on their chemical shifts, the C-1 signals at δ 98.00, 98.31, and 99.66 were assigned to three α-hexopyranosyl residues (**C**, **D**, and **E**), whereas the C-1 signals

β-D-Gal*f* F
1
↓
3
→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→ 1
A B C D E

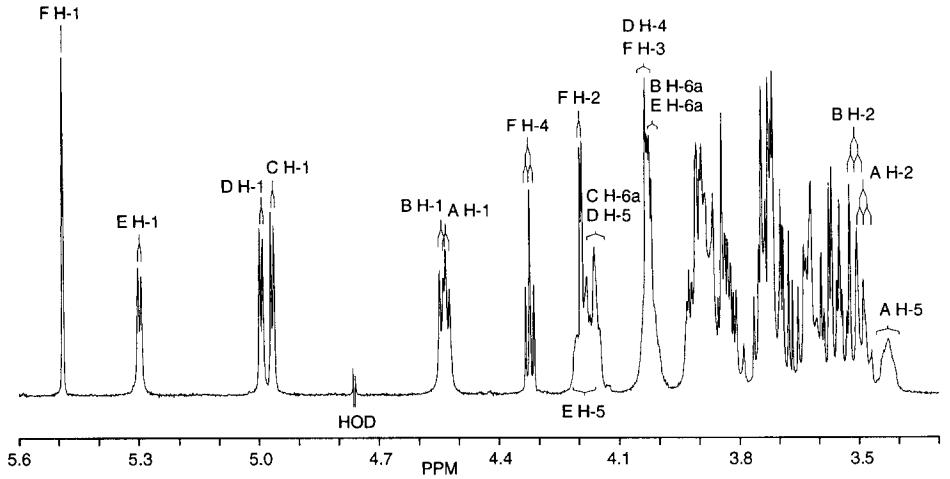


Fig. 1. 500-MHz ^{1}H NMR spectrum of native EPS (**1**), recorded in D_2O at 27°C.

at δ 102.13 and 102.75 were assigned to two β-hexopyranosyl residues (**A** and **B**). The downfield signal at δ 107.80 was attributed to C-1 of the galactofuranosyl group **F** and its anomeric configuration was provisionally assigned as β (Me-β-D-Gal*f*, $\delta_{C\text{-}1}$ 109.9; Me-α-D-Gal*f*, $\delta_{C\text{-}1}$ 103.8 [16]). The resonances at δ 60.70 and 60.01 were assigned to hydroxymethyl carbons (unsubstituted C-6) of hexopyranosyl residues, and the signal at δ 63.14 to C-6 of the galactofuranosyl group **F** (Me-β-D-Gal*f*, $\delta_{C\text{-}6}$ 63.6; Me-α-D-Gal*f*, $\delta_{C\text{-}6}$ 64.1 [16]).

Partial acid hydrolysis.—The complex mixture of oligosaccharides, obtained after partial acid hydrolysis of **1** (elution profile on CarboPac PA-1, Fig. 2A), was preparatively fractionated on Bio-Gel P-2, yielding fractions I to V (Fig. 2A). Fraction I contained only galactose (~ 90%) and glucose (~ 10%), as demonstrated by GLC and ^{1}H NMR spectroscopy. Fractions II to V were subfractionated on CarboPac PA-1 (Fig. 2B), and the resulting HPAEC-fractions **3**–**11** were reduced with $NaBD_4$, prior to further analysis. After reduction, a further fractionation of fractions **8**–**11** on CarboPac PA-1 was performed, yielding subfractions **8a**–**11a** (Fig. 2C).

Fractions **3** *to* **7**.—According to monosaccharide analysis, methylation analysis, and 1D/2D NMR spectroscopy, each of the reduced fractions **3**–**7** was shown to contain a single disaccharide-alditol-*1-d*. A survey of the structures is presented in Fig. 3. ^{1}H NMR chemical shifts and coupling constants of the disaccharide-alditols-*1-d* **3**–**7** are listed in Table 2. The 1D ^{1}H NMR spectra of **3**, **5**, **6**, and **7** are identical to the spectra of reference melibiitol-*1-d*, gentiobiitol-*1-d*, cellobiitol-*1-d*, and nigeritol-*1-d*, respectively.

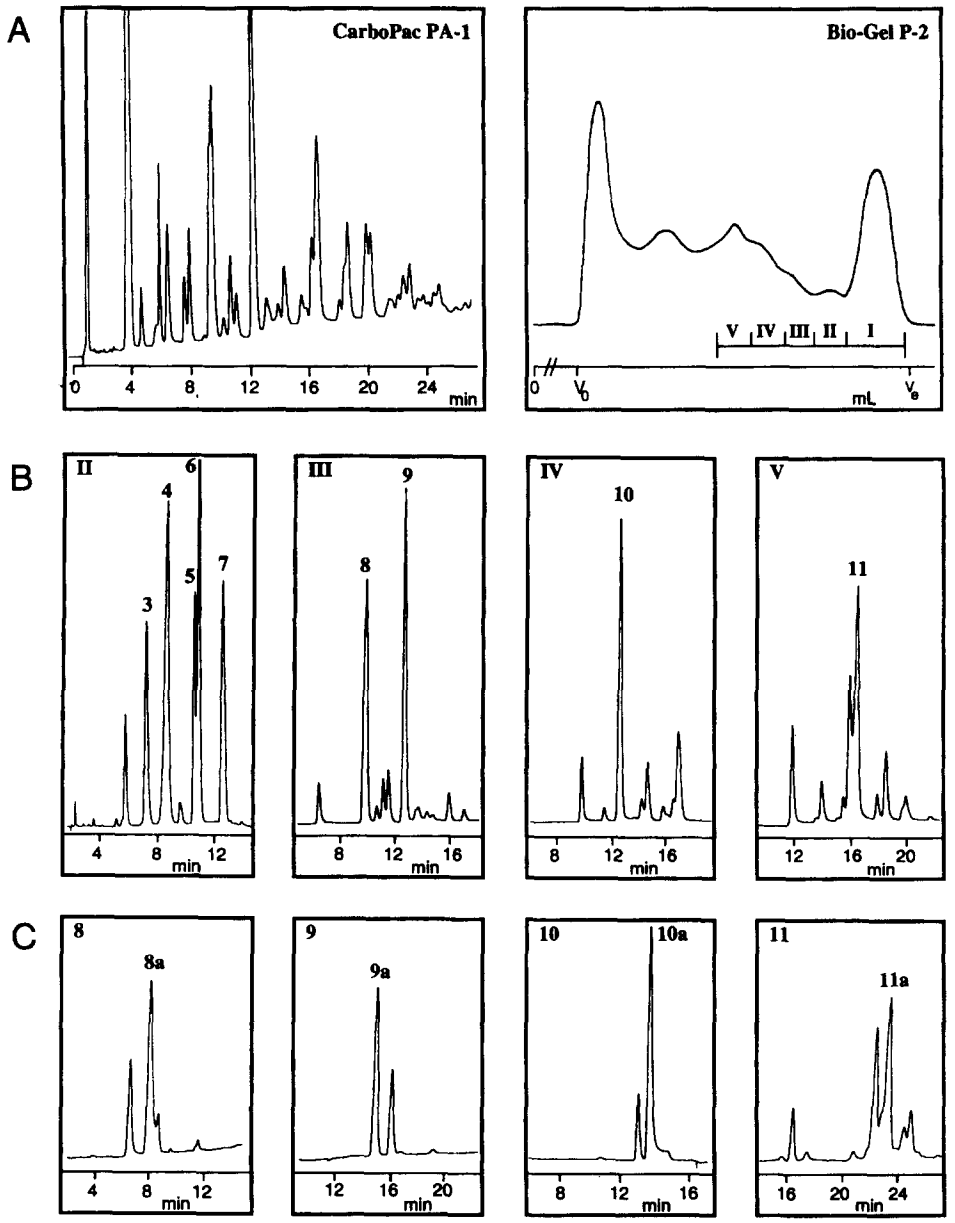


Fig. 2. (A) HPAEC-PAD (analytical run) and Bio-Gel P-2 (preparative run) elution patterns of a partial acid hydrolysate of native EPS. (B) Preparative HPAEC-PAD fractionation patterns of Bio-Gel P-2 fractions II–V. (C) Preparative HPAEC-PAD fractionation patterns of fractions **8**–**11**, after reduction with $NaBD_4$.

Fraction **8**.—The major component (**8a**) of reduced fraction **8** had a monosaccharide composition of glucose, galactose, and glucitol in molar ratios of 2:1:1. Methylation analysis of **8a** demonstrated the presence of terminal and 6-substituted glucopyranose,

α-D-Gal*p*-(1→6)-D-Glc-ol-*1-d* **3**

α-D-Glc*p*-(1→6)-D-Gal-ol-*1-d* **4**

β-D-Glc*p*-(1→6)-D-Glc-ol-*1-d* **5**

β-D-Glc*p*-(1→4)-D-Glc-ol-*1-d* **6**

α-D-Glc*p*-(1→3)-D-Glc-ol-*1-d* **7**

β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc-ol-*1-d* **8a**
B **C** **D** **E**-ol

β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Gal-ol-*1-d* **9a**
A **B** **C** **D**-ol

β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc-ol-*1-d* **10a**
A **B** **C** **D** **E**-ol

α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Gal-ol-*1-d* **11a**
E **A** **B** **C** **D**-ol

β-D-Gal*f* **F**
1
↓
3
→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→ **1**
A **B** **C** **D** **E**

Fig. 3. Structures of oligosaccharide-alditols **3–11a** obtained by partial acid hydrolysis of the native polysaccharide, and the structure of the repeating unit of native EPS (**1**).

6-substituted galactopyranose, and 6-substituted glucitol-*1-d* in approximately equimolar amounts, indicating a linear tetrasaccharide-alditol. In the high-mass region of the positive-ion mode FAB mass spectrum of permethylated **8a**, a strong $[M + Na]^+$ pseudomolecular ion at m/z 902 was detected, corresponding to a composition of Hex_3Hex-ol-*1-d*. In the 1D ^1H NMR spectrum of **8a** (Fig. 4A) three anomeric signals are observed at δ 4.492 (residue **B**, $^3J_{1,2}$ 7.9 Hz), δ 4.944 (residue C, $^3J_{1,2}$ 3.8 Hz), and δ 4.975 (residue **D**, $^3J_{1,2}$ 3.8 Hz), indicating the presence of one β- and two α-hexopyranosyl residues. The complete proton assignments for residues **B**, **C**, and **D**, as presented in Table 3, are based on cross-peaks observed in the 2D COSY, HOHAHA, and ROESY spectra of **8a**. Owing to the reduction with $NaBD_4$, **8a** was obtained as a mixture of two isotopic diastereomers (labeled a and b). Consequently, different

Table 2
^{1}H NMR chemical shifts [a] of disaccharide-alditols-*1-d* **3–7**, obtained from a partial acid hydrolysate of native EPS, recorded at 27°C. Coupling constants (Hz) are given in parentheses

Compound	Residue	H-1(a) [b]	H-1(b) [b]	H-2	H-3	H-4	H-5	H-6a	H-6b
3	Gal-α-(1 →	4.981 (3.7)	–	3.835	3.895	3.991	3.98	3.743	3.743
	→ 6)-Glc-ol	3.607 (6.3)	3.716	3.835	3.865	3.727	3.920	3.682 (2.4, −10.5)	3.900
4	Glc-α-(1 →	4.960 (3.7)	–	3.566 (9.8)	3.74	3.417 (~9.4)	3.74	3.87 [c]	3.775 [c]
	→ 6)-Gal-ol	3.693	3.693	3.974	3.672	3.740	4.149	3.850 [d]	3.650 [d]
5	Glc-β-(1 →	4.498 (7.9)	–	3.325 (9.5)	3.505 (9.2)	3.391 (9.3)	3.465	3.921 (2.3, −12.3)	3.722 (6.0)
	→ 6)-Glc-ol	3.601 (6.4)	3.720	3.845	3.850	3.735	3.925	4.150 (2.6, −11.0)	3.815
6	Glc-β-(1 →	4.569 (7.9)	–	3.333 (9.3)	3.503 (9.1)	3.429 (9.6)	3.435	3.907 (~2, −12.5)	3.768 (5.1)
	→ 4)-Glc-ol	3.624 (6.9)	3.760	3.952	3.842	3.870 (7.0)	3.87	3.945	3.744 (6.0, −11.7)
7	Glc-α-(1 →	5.136 (3.9)	–	3.569 (10.0)	3.738 (9.3)	3.440 (9.2)	3.81	3.85 [e]	3.78 [e]
	→ 3)-Glc-ol	3.697 (6.8)	3.800	3.980 (5.5)	3.995 (1.8)	3.770 (7.7)	3.865	3.825	3.653 (6.1, −11.7)

[a] In ppm relative to the signal of internal acetone at δ 2.225.
[b] The assignments of H-1 of the two isotopic diastereomers [H-1(a) and H-1(b)] may have to be interchanged within one compound.
[c–e] The assignments of H-6a and H-6b may have to be interchanged within one residue.

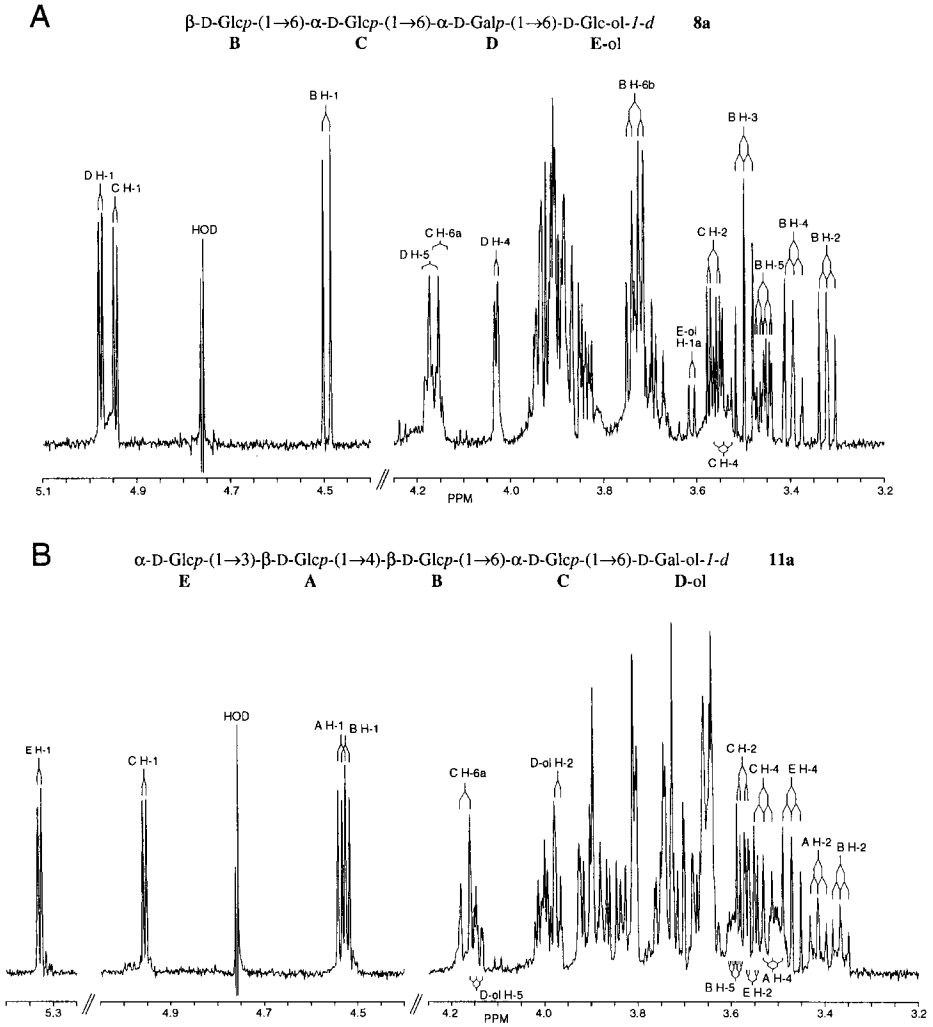


Fig. 4. 500-MHz ^{1}H NMR spectra of (A) tetrasaccharide-alditol-*1-d* **8a** and (B) pentasaccharide-alditol-*1-d* **11a**, recorded in D_2O at 27°C.

chemical shifts might be expected for **E**-ol H-1 in both compounds a and b. Starting from the well-resolved doublet at δ 3.613 ($^3J_{1a,2}$ 6.3 Hz, intensity ~ 0.3 H), assigned to **E**-ol H-1(a), **E**-ol H-2 was detected in the COSY spectrum, and from **E**-ol H-2 the resonances of **E**-ol H-1(b) and **E**-ol H-3 were found. In the ROESY spectrum an interresidual connectivity **D** H-1,**E**-ol H-6b is observed at δ 3.68. On the **E**-ol H-6b track in the HOHAHA spectrum, cross-peaks with **E**-ol H-6a,5,4,3 are observed, thus completing the assignment of residue **E**-ol. In addition to the ROE cross-peak **D** H-1,**E**-ol H-6b, which confirms the **D**(1 → 6)**E**-ol sequence, interresidual ROE connec-

Table 3
^{1}H NMR chemical shifts [a] of native EPS (**1**), and oligosaccharide-alditols-*1-d* **8a**, **9a**, **10a**, and **11a**, obtained from a partial acid hydrolysate of **1**, recorded at 27°C. Coupling constants (Hz) are given in parentheses

Residue		**1**	**8a**	**9a**	**10a**	**11a**
A	H-1	4.528 (7.7)	–	4.509 (7.7)	4.510 (7.9)	4.534 (8.0)
	H-2	3.482	–	3.313 (9.2)	3.314 (9.2)	3.414 (9.1)
	H-3	3.625	–	3.507 (9.3)	3.509 (9.3)	3.65 (~ 9.4)
	H-4	3.625	–	3.416 (9.5)	3.416 (9.4)	3.511 (~ 9.4)
	H-5	3.424	–	3.488	3.50	3.63
	H-6a [b]	3.92	–	3.915	3.915	3.91
	H-6b [b]	3.73	–	3.73	3.73	3.73
B	H-1	4.537 (7.8)	4.492 (7.9)	4.524 (7.7)	4.518 (8.0)	4.525 (8.0)
	H-2	3.502	3.320 (9.3)	3.364 (8.8)	3.367 (8.6)	3.366 (9.4)
	H-3	3.867	3.500 (9.2)	3.65	3.65	3.65
	H-4	3.809	3.393 (9.7)	3.65	3.65	3.65
	H-5	3.61	3.456	3.59	3.59	3.59
	H-6a	4.02	3.92 (2.3, −12.0)	3.985 (−12.3)	3.988 (2.1, −12.4)	3.985
	H-6b	3.87	3.731 (5.9)	3.821 (5.1)	3.82	3.82
C	H-1	4.963 (3.7)	4.944 (3.8)	4.955 (3.7)	4.943 (3.7)	4.956 (3.7)
	H-2	3.564	3.563 (9.8)	3.574 (9.8)	3.569 (9.5)	3.574 (~ 9.8)
	H-3	3.717	3.715 (9.3)	3.74 (9.4)	3.72	3.74 (9.4)
	H-4	3.55	3.544 (9.3)	3.532 (9.4)	3.54	3.532 (9.4)
	H-5	3.88	3.89	3.88	3.89	3.88
	H-6a [c]	4.17	4.152	4.17	4.16	4.17
	H-6b [c]	3.90	3.91	3.91	3.91	3.91
D	H-1	4.991 (3.7)	4.975 (3.8)	–	4.975 (3.8)	–
	H-2	3.82	3.825	–	3.825	–
	H-3	3.90	3.91 (3.7)	–	3.91 (3.4)	–
	H-4	4.025	4.029	–	4.025	–
	H-5	4.16	4.172	–	4.18	–
	H-6a [d]	3.89	3.88	–	3.88	–
	H-6b [d]	3.73	3.72	–	3.72	–
E	H-1	5.295 (3.8)	–	–	–	5.329 (3.9)
	H-2	3.58	–	–	–	3.588 (9.8)
	H-3	3.74	–	–	–	3.74 (9.6)
	H-4	3.515	–	–	–	3.470 (9.7)
	H-5	4.20	–	–	–	4.00
	H-6a [e]	4.01	–	–	–	3.80
	H-6b [e]	3.71	–	–	–	3.80
F	H-1	5.488 (< 2)	–	–	–	–
	H-2	4.192 (2.6)	–	–	–	–
	H-3	4.03 (5.3)	–	–	–	–
	H-4	4.320 (5.1)	–	–	–	–
	H-5	3.83	–	–	–	–
	H-6a	3.73 (~ 5, ~ 11)	–	–	–	–
	H-6b	3.67 (~ 7)	–	–	–	–
D-ol	H-1(a)	–	–	3.675	–	3.675
	H-1(b)	–	–	3.675	–	3.675
	H-2	–	–	3.970	–	3.975
	H-3	–	–	3.688	–	3.690
	H-4	–	–	3.740	–	3.740
	H-5	–	–	4.150	–	4.150
	H-6a [f]	–	–	3.860	–	3.865
	H-6b [f]	–	–	3.650	–	3.650

Table 3 (continued)

Residue		1	8a	9a	10a	11a
E-ol	H-1(a)	–	3.613 [h] (6.3)	–	3.61 [i]	–
	H-1(b)	–	3.71 [h]	–	3.715 [i]	–
	H-2	–	3.84	–	3.84	–
	H-3	–	3.87	–	3.87	–
	H-4	–	3.725	–	3.73	–
	H-5	–	3.93	–	3.92	–
	H-6a [g]	–	3.91	–	3.91	–
	H-6b [g]	–	3.68	–	3.68	–

[a] In ppm relative to the signal of internal acetone at δ 2.225.
[b–g] The assignments of H-6a and H-6b may have to be interchanged within one residue.
[h,i] The assignments of H-1 of the two isotopic diastereomers [H-1(a) and H-1(b)] may have to be interchanged within one compound.

tivities **B** H-1,**C** H-6b and **C** H-1,**D** H-6b are observed, supporting the **B**(1 → 6)**C**(1 → 6)**D** sequence, and thus proving the structure of **8a** as presented in Fig. 3.

Fraction **9**.—1D ^{1}H NMR analysis of reduced fraction **9** suggested that it was composed of a mixture of oligosaccharide-alditols. In the high-mass region of the positive-ion mode FAB mass spectrum of reduced and permethylated fraction **9** two strong $[M + Na]^+$ pseudomolecular ions at m/z 902 (relative abundance 100) and m/z 1106 (relative abundance 20) were observed, corresponding to compositions of Hex_3Hex-ol-*1-d* and Hex_4Hex-ol-*1-d*, respectively. The 1D ^{1}H NMR spectrum of the minor component, obtained after subfractionation of reduced fraction **9** on CarboPac PA-1 (Fig. 2C), was identical to the spectrum of pentasaccharide-alditol-*1-d* **10a** (vide infra). The major component, **9a**, had a monosaccharide composition of glucose and galactitol in a molar ratio of 3:1. Methylation analysis revealed the presence of terminal, 4-substituted, and 6-substituted glucopyranose, and 6-substituted galactitol-*1-d* in equimolar amounts, suggesting a linear tetrasaccharide-alditol. In the 1D ^{1}H NMR spectrum of **9a** (not shown) three anomeric signals are present at δ 4.509 (residue **A**, $^3J_{1,2}$ 7.7 Hz), δ 4.524 (residue **B**, $^3J_{1,2}$ 7.7 Hz), and δ 4.955 (residue **C**, $^3J_{1,2}$ 3.7 Hz), reflecting two β- and one α-hexopyranosyl residues. All non-anomeric resonances for the residues **A, B**, and **C** were found starting from the anomeric signals in the 2D COSY and HOHAHA spectra (Table 3). A characteristic multiplet at δ 4.150 (ddd), assigned to **D**-ol H-5 [17], was used as starting point for the assignment of the resonances of residue **D**-ol. On the **D**-ol H-5 HOHAHA track, cross-peaks with **D**-ol H-6a,6b,4 were found, and connectivities in the HOHAHA and COSY spectra allowed the identification of **D**-ol H-3,2,1. The interresidual connectivity **C** H-1,**D**-ol H-6b in the ROESY spectrum confirms the **C**(1 → 6)**D**-ol sequence. The interresidual ROE cross-peaks **A** H-1,**B** H-4 and **B** H-1,**C** H-6b establish the **A**(1 → 4)**B**(1 → 6)**C** sequence, proving the structure of **9a** as given in Fig. 3.

Fraction **10**.—The 1D ^{1}H NMR spectrum of reduced fraction **10** indicated a mixture of oligosaccharide-alditols. In the high-mass region of the positive-ion mode FAB mass spectrum of reduced and permethylated fraction **10** two strong $[M + Na]^+$ pseudomolecular ions at m/z 902 (relative abundance 80) and m/z 1106 (relative abundance 100)

are observed, corresponding to compositions of Hex_3Hex-ol-*1-d* and Hex_4Hex-ol-*1-d*, respectively. The minor component, obtained after subfractionation of reduced fraction **10** on CarboPac PA-1 (Fig. 2C), was determined to be identical to tetrasaccharide-alditol-*1-d* **9a** by 1D ^{1}H NMR analysis (vide supra). Monosaccharide analysis of the major component, **10a**, revealed a composition of glucose, galactose, and glucitol in molar ratios of 3:1:1. Methylation analysis indicated the presence of terminal, 4-substituted, and 6-substituted glucopyranose, 6-substituted galactopyranose, and 6-substituted glucitol-*1-d* in equimolar amounts, suggesting a linear pentasaccharide-alditol. In the 1D ^{1}H NMR spectrum of **10a** (not shown), four anomeric signals appear at δ 4.510 (residue **A**, $^3J_{1,2}$ 7.9 Hz), δ 4.518 (residue **B**, $^3J_{1,2}$ 8.0 Hz), δ 4.943 (residue **C**, $^3J_{1,2}$ 3.7 Hz), and δ 4.975 (residue **D**, $^3J_{1,2}$ 3.8 Hz), indicating two β- and two α-hexopyranosyl residues. Starting from the anomeric signals in the 2D COSY and HOHAHA spectra, all non-anomeric resonances for the residues **A**, **B**, **C**, and **D** were detected (Table 3). In the ROESY spectrum of **10a** a strong interresidual cross-peak **D** H-1,**E**-ol H-6b (δ 3.68) is observed, reflecting the **D**(1 → 6)**E**-ol sequence, which was used as starting point for the assignment of the **E**-ol subspectrum. By comparison with chemical shifts of the alditol-*1-d* residues in the oligosaccharide-alditols **3** and **8a**, all resonances for **E**-ol could be traced in the COSY and HOHAHA spectra. In the ROESY spectrum, the interresidual connectivities **A** H-1,**B** H-4 and **B** H-1,**C** H-6b confirm the **A**(1 → 4)**B**(1 → 6)**C** sequence. On the **C** H-1 track, interresidual connectivities are observed with **D** H-6a (weak) and **D** H-6b (strong), corroborating the **C**(1 → 6)**D** sequence. The combined evidence established the structure of **10a** as given in Fig. 3.

Fraction **11**.—The major component (**11a**) of reduced fraction **11** had a monosaccharide composition of glucose and galactitol in a molar ratio of 4:1. Methylation analysis demonstrated the presence of terminal, 3-substituted, 4-substituted, and 6-substituted glucopyranose and 6-substituted galactitol-*1-d* in approximately equimolar amounts, indicating a linear pentasaccharide-alditol. In the high-mass region of the positive-ion mode FAB mass spectrum of permethylated **11a**, a strong $[M + Na]^+$ pseudomolecular ion was observed at m/z 1106, corresponding to a composition of Hex_4Hex-ol-*1-d*. The 1D ^{1}H NMR spectrum of **11a** (Fig. 4B) shows four anomeric signals at δ 4.534 (residue **A**, $^3J_{1,2}$ 8.0 Hz), δ 4.525 (residue **B**, $^3J_{1,2}$ 8.0 Hz), δ 4.956 (residue **C**, $^3J_{1,2}$ 3.7 Hz), and δ 5.329 (residue **E**, $^3J_{1,2}$ 3.9 Hz), indicating the presence of two β- and two α-hexopyranosyl residues. Starting from the anomeric signals, via the COSY and HOHAHA spectra all non-anomeric signals for the residues **E, A, B**, and **C** could be assigned (Table 3). Analogous to oligosaccharide-alditol **9a**, for residue **D**-ol the multiplet at δ 4.150 (ddd), assigned to **D**-ol H-5 [17], was used as starting point for the determination of the other **D**-ol resonances. In the ROESY spectrum the interresidual connectivity **E** H-1,**A** H-3 confirms the **E**(1 → 3)**A** sequence. Because of the small amount of carbohydrate material, the ROESY experiment was not sensitive enough to establish the sequence of the other residues in **11a**. However, comparison of the complete set of ^{1}H NMR chemical shifts of **11a** to those of **9a** strongly suggests that the two oligosaccharides are identical except for an extra (1 → 3)-linked α-D-Glc*p*-unit at the non-reducing end of **11a**. Therefore the structure of **11a** is as presented in Fig. 3.

Taking into account that the backbone of the native EPS is composed of pentasaccharide repeating units (as determined by 1D NMR spectroscopy and methylation analysis,

Table 4
^{13}C NMR chemical shifts [a] of native EPS (**1**) as determined from the 1D ^{13}C spectrum, recorded at 75.469 MHz, at a probe temperature of 27°C. The assignments were deduced from a 2D $^{13}C-^{1}H$ HMQC experiment, recorded at the same temperature. $^{1}J_{C\text{-}1,H\text{-}1}$ values (Hz) are given in parentheses

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	102.13 (162)	72.53	83.72	69.94	75.99	60.70
B	102.75 (162)	74.09	77.64	73.93	75.52	60.01
C	98.00 (170)	71.50	73.23	69.42	71.03	68.52
D	98.31 (171)	68.77	69.76	69.67	68.98	66.90
E	99.66 (172)	71.82	73.36	69.88	70.74	66.08
F	107.80 (178)	81.02	77.93	84.39	71.50	63.14

[a] In ppm relative to the signal of external methanol at δ 49.00.

vide supra), the combination of the structural information from the oligosaccharide-alditols **3–11a** allowed structure **12** to be formulated as the structure for the polysaccharide backbone:

→ 3)-β-D-Glc*p*-(1 → 4)-β-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-α-D-Glc*p*-(1 → **12**
A **B** **C** **D** **E**

2D NMR spectroscopy on the native EPS.—The combination of the methylation analysis data (native EPS, before and after mild acid hydrolysis) and the structure of the backbone of the EPS, **12**, as proposed above, indicated that the EPS (**1**) is composed of branched hexasaccharide repeating units, as presented in Fig. 3. To verify this conclusion, and to be able to exclude the presence of any other substituents that may not have been detected by the degradative studies discussed so far, **1** was also examined independently by 2D NMR experiments. The ^{1}H NMR data are presented in Table 3, and the ^{13}C NMR data are given in Table 4.

All ^{1}H resonances in the 1D ^{1}H NMR spectrum of **1** (Fig. 1) were assigned by means of 2D COSY and HOHAHA experiments (spectra not shown). On the HOHAHA track of **A** H-1 at δ 4.528, the complete series of cross-peaks with **A** H-2,3,4,5,6a,6b is observed. The resonances for **B** H-2,3,4,5,6b were found via strong connectivities on the **B** H-1 track (δ 4.537), whereas **B** H-6a is observed as a less intense cross-peak on the **B** H-1 track, and via connectivities with **B** H-5 and **B** H-6b in the COSY spectrum. On the HOHAHA **C** H-1 track (δ 4.963) cross-peaks with **C** H-2,3,4,5 are observed, and the resonances for **C** H-6a,6b were determined starting from **C** H-5 in the COSY spectrum. The HOHAHA **D** H-1 track (δ 4.991) shows connectivities with **D** H-2,3,4 and the resonances of **D** H-5,6a,6b were assigned starting from **D** H-5 in the COSY spectrum. Although the connectivity **D** H-4,5 could not be observed in the COSY and HOHAHA spectra (due to the small value of $^{3}J_{4,5}$ for the galactopyranosyl residue **D**), such a connectivity was detected in the NOESY spectrum (Fig. 6), thereby linking the two subsets of ^{1}H resonances **D** H-1,2,3,4 and **D** H-5,6a,6b. On the HOHAHA **E** H-1 track, cross-peaks with **E** H-2,3,4,5 were detected and the resonances for **E** H-6a,6b were found on the HOHAHA **E** H-5 track. Finally, the subspectrum of the galactofuranosyl group **F** was assigned via connectivities with **F** H-2,3,4 on the **F** H-1 track at δ 5.488

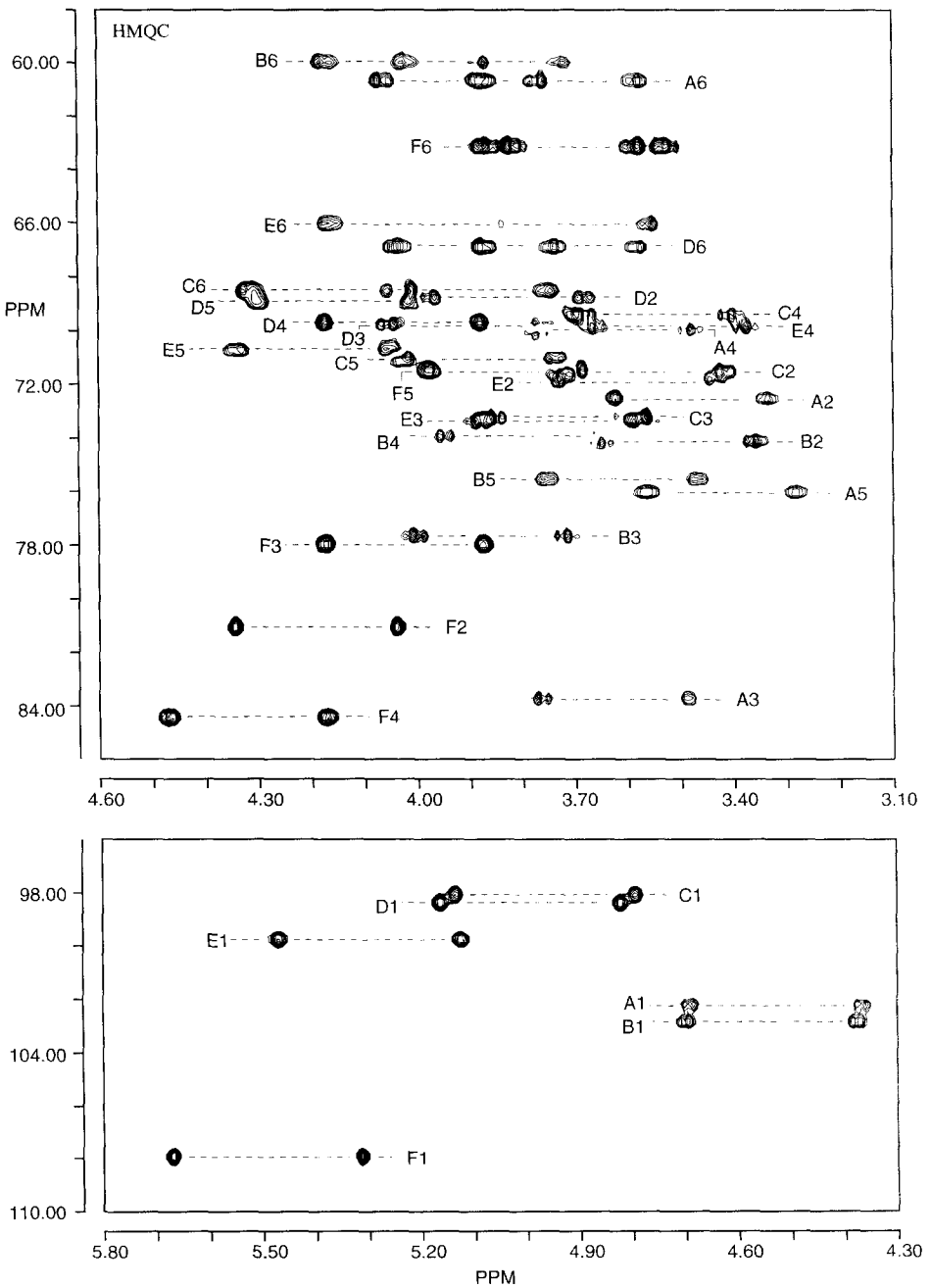


Fig. 5. 500-MHz 2D $^{13}C-^{1}H$ HMQC spectrum of native EPS (1), recorded in D_2O at 27°C. Al denotes the set of cross-peaks between H-1 and C-1 of residue A, etc.

and via cross-peaks with **F** H-5,6a,6b on the **F** H-4 track at δ 4.320 in the HOHAHA spectrum.

The 2D $^{13}C-^{1}H$ HMQC spectrum of **1** (Fig. 5) allowed the complete assignment of its ^{13}C spectrum. The observed $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants (Table 4) of 162 Hz for the residues **A** and **B** and 170–172 Hz for the residues **C**, **D**, and **E** confirm the assignments of their anomeric configurations as β and α, respectively. The $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constant for residue **F** (178 Hz) is indicative of a furanose ring form, although it gives no information about the anomeric configuration of the residue [18]. However, comparison of the complete set of ^{13}C chemical shifts for residue **F** with the sets of Me-β-D-Gal*f* ($\delta_{C\text{-}1}$ 109.9, $\delta_{C\text{-}2}$ 81.3, $\delta_{C\text{-}3}$ 78.4, $\delta_{C\text{-}4}$ 84.7, $\delta_{C\text{-}5}$ 71.7, $\delta_{C\text{-}6}$ 63.6) and Me-α-D-Gal*f* ($\delta_{C\text{-}1}$ 103.8, $\delta_{C\text{-}2}$ 78.2, $\delta_{C\text{-}3}$ 76.2, $\delta_{C\text{-}4}$ 83.1, $\delta_{C\text{-}5}$ 74.5, $\delta_{C\text{-}6}$ 64.1) [16] proves the β configuration. The ^{13}C NMR chemical shifts of **C** C-6 (δ 68.52), **D** C-6 (δ 66.90), and **E** C-6 (δ 66.08) indicate that the residues **C**, **D** and **E** are 6-substituted, whereas the chemical shifts of **A** C-6 (δ 60.70) and **B** C-6 (δ 60.01) confirm that these residues are not 6-substituted. The relatively downfield chemical shift of **A** C-3 (δ 83.72) proves that residue **A** is 3-substituted, and the chemical shifts of **B** C-3 (δ 77.64) and **B** C-4 (δ 73.93) indicate that residue **B** is 3,4-disubstituted (Me-β-D-Glc*p*, $\delta_{C\text{-}3}$ 76.8, $\delta_{C\text{-}4}$ 70.6 [16]).

Recently, a computational approach has been developed by Lipkind et al. for the determination of primary structures of polysaccharides using ^{13}C NMR chemical shifts [19–21]. This method employs ^{13}C NMR glycosylation-increment tables to calculate ^{13}C NMR spectra for different possible structures. The calculated spectra are then compared to the observed spectrum and the fit for each of the theoretical spectra is expressed by the fit-value *S*, which is defined as $\Sigma\Delta^2/n$, in which Δ is the difference between the observed and the calculated chemical shifts for a given carbon in the polysaccharide, and *n* represents the number of monosaccharides. An *S*-value not exceeding 1.50 represents a good fit.

This approach is suitable for linear polysaccharides, as well as for a limited number of branched polysaccharides. However, the method is not capable of dealing with galactofuranosyl side chains. Therefore, in our case, the increment tables of the method were applied to calculate the theoretical ^{13}C NMR spectrum for the backbone of **1** (i.e. without the galactofuranosyl side chain). When the calculated spectrum (backbone) is compared with the observed ^{13}C NMR spectrum (native EPS) only the values of **B** C-3 (δ_{calc} 74.45; δ_{obs} 77.64; $\Delta\delta$ −3.19) and **B** C-4 (δ_{calc} 78.75; δ_{obs} 73.93; $\Delta\delta$ +4.82) differ significantly, which is in accordance with the substitution of residue **B** by residue **F** at **B** O-3. When all of the backbone resonances, except **B** C-3 and C-4, are considered, the fit to the calculated spectrum is good, with an *S*-value of 1.32.

The 2D NOESY spectrum of **1** (Fig. 6) confirms the anomeric configurations and the sequence of the different monosaccharide residues in the polysaccharide. The observed intraresidual NOE contacts **F** H-1,H-2 and **F** H-2,H-3 are in agreement with the small $^{3}J_{H\text{-}1,H\text{-}2}$ and $^{3}J_{H\text{-}2,H\text{-}3}$ coupling constants (< 2 Hz and 2.6 Hz, respectively), suggesting an envelope ring conformation in which the torsion angles $\phi_{1,2}$ and $\phi_{2,3}$ are close to 90° [22]. The interresidual NOE connectivity **F** H-1,**B** H-3 is in agreement with the substitution of residue **B** by the galactofuranosyl group **F** at **B** O-3. The interresidual connectivities **A** H-1,**B** H-4 and **B** H-1,**C** H-6b confirm the **A**(1 → 4)**B**(1 → 6)**C**

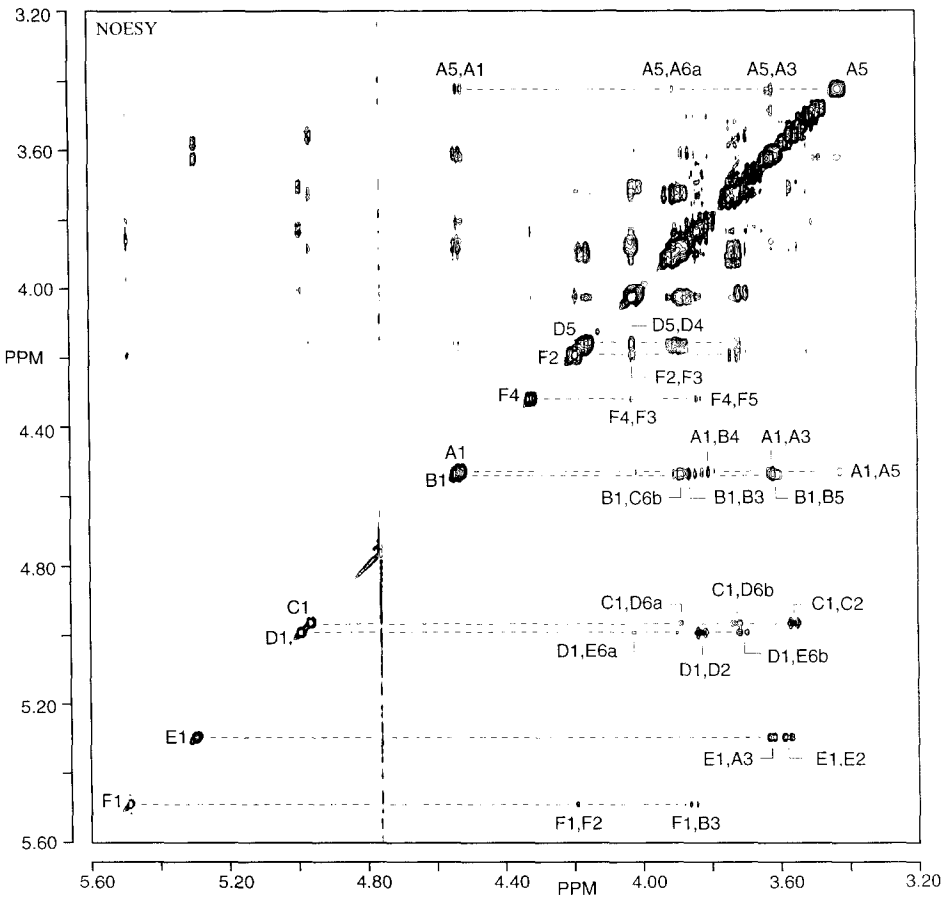
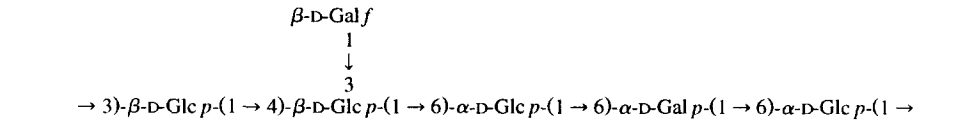


Fig. 6. 500-MHz 2D NOESY spectrum (mixing time 200 ms) of native EPS (**1**), recorded in D_2O at 27°C. F1 corresponds to the diagonal peak belonging to **F** H-1; F1,B3 refers to a connectivity between **F** H-1 and **B** H-3, etc.

sequence. From the connectivities **C** H-1,**D** H-6a (weak), **C** H-1,**D** H-6b (strong), **D** H-1,**E** H-6a (weak), and **D** H-1,**E** H-6b (strong), the sequence **C**(1 → 6)**D**(1 → 6)**E** was established and, finally, the strong NOE connectivity **E** H-1,**A** H-3 demonstrated the **E**(1 → 3)**A** linkage.

In conclusion, the evidence obtained along the different independent approaches, as described, demonstrates that the EPS produced by *Lactobacillus helveticus* 766 in skimmed milk is composed of hexasaccharide repeating units with the following structure:



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