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Structure of the exopolysaccharide produced by *Lactococcus lactis* subspecies *cremoris* H414 grown in a defined medium or skimmed milk

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ABSTRACT

The structure of the exopolysaccharide of *Lactococcus lactis* subsp. *cremoris* H414, isolated from a defined medium or skimmed milk, was established by linkage analysis on the native polysaccharide, and by characterisation of oligosaccharide fragments, obtained by Smith degradation and partial acid hydrolysis, using methylation analysis, FABMS, EIMS, and ^{1}H-NMR spectroscopy. The polysaccharide has the branched-pentasaccharide repeating unit:

→ 4)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-[β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)]-α-D-Gal*p*-(1 →.

INTRODUCTION

Many lactic acid bacteria produce exocellular polysaccharides that are of commercial interest because of the viscosity of their aqueous solutions. In order to understand the rheology of these polysaccharides, a knowledge of the structures is a prerequisite. The extracellular polysaccharides can be homo, e.g., the dextrans formed by the genus *Leuconostoc* and the mutans formed by "mutans" streptococci[1], or hetero in character, e.g., those produced by *Streptococcus thermophilus*[2], *Lactobacillus helveticus* var. *yugurti*[3], and *L. bulgaricus*[4].

For *Lactococcus lactis* subsp. *cremoris*, the slime of type 351 has[5] a carbohydrate content of 85% with Gal and Glc in the molar ratio 2:1. The slime of type L416 is a glycoprotein[6] that contains protein (47%), hexoses (9.3%), 6-deoxyhexoses (20%), and sialic acid (2.8%). The main component[7] of the slime of type SBT

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0495 is a phosphopolysaccharide with Rha, Glc, and Gal in the molar ratios 1:1.45:1.75. We now report the purification and characterisation of the exopolysaccharide produced by strain H414 (VBSSt21-12).

EXPERIMENTAL

Growth of the organism and isolation of the exopolysaccharide.—(*a*) *L. lactis* subsp. *cremoris* strain H414 was cultured in skimmed milk for 16 h at 20°, trichloroacetic acid was added to 4%, and the bacterial cells and precipitated proteins were removed by centrifugation (20 min, 13 000*g*, 4°). Acetone (1 vol) was added to the supernatant solution, and the precipitated polysaccharide was collected, dialysed against running tap water (48 h) and bidistilled water (24 h, 5 × 5 L), and then lyophilised.

(*b*) Strain H414 was cultured in a stirred fermenter under N_2 in a defined medium[8,9] with lactose as the carbon source for 48 h at 30° and pH 7.0. The bacterial cells were removed by centrifugation (20 min, 13 000*g*, 4°), acetone (2 vol) was added to the supernatant solution, and the precipitated polysaccharide material was collected as in (*a*).

Each lyophilised polysaccharide was dissolved in bidistilled water and, after removal of insoluble material by centrifugation (30 min, 30 000*g*, 4°), fractionally precipitated with acetone at 30, 40, 50, 60, and 70% with intermediate centrifugation. The exopolysaccharide was precipitated at 40% acetone, and, after lyophilisation, contaminating protein was removed by gel filtration on a column (150 × 2.2 cm) of Sephacryl S-500 (Pharmacia) by elution with 50 mM NH_4HCO_3 at 25 mL/h (5-mL fractions), with refractive-index monitoring (Bischoff RI-detector 8100) of the eluate.

Analytical methods.—Samples (0.2–0.5 mg) were methanolysed (methanolic M HCl, 24 h, 85°), and the trimethylsilylated (*N*-reacetylated) methyl glycosides were analysed on an SE-30 fused-silica capillary column (25 m × 0.32 mm, Pierce), using a Varian 3700 gas chromatograph (temperature program 130 → 220° at 4°/min)[10]. The absolute configuration of the monosaccharides was determined by GLC of the trimethylsilylated (*N*-reacetylated) (−)-2-butyl glycosides[11,12].

Protein content was determined with the Pierce Protein Assay Reagent with bovine serum albumin as the standard.

Total carbohydrate contents were measured by the phenol–H_2SO_4 assay[13] with D-galactose as the standard.

Methylation analysis.—Polysaccharide and oligosaccharide-alditols were each methylated according to Kvernheim[14], and the products were hydrolysed with aq 90% formic acid (1 h, 100°), then 2 M trifluoroacetic acid (1 h, 120°). The resulting partially methylated monosaccharides were reduced with $NaBD_4$ and the products were acetylated[15]. The partially methylated alditol acetates were analysed by GLC on a CPsil 43 WCOT fused-silica capillary column (25 m × 0.32 mm, Chrompack),

using a Varian 3700 gas chromatograph (temperature program 170 → 220° at 4°/min, followed by 15 min at 220°), and by GLC–MS using a Carlo Erba GC/Kratos MS80/Kratos DS 55 system (electron energy, 70 eV; accelerating voltage, 2.7 kV; ionising current, 100 mA; CPsil 43 capillary column).

Smith degradation.—To a solution of the polysaccharide (50 mg) in 0.1 M NaOAc buffer (50 mL, pH 3.9) was added sodium metaperiodate to 0.05 M, and the solution was kept in the dark for 5 days at 4°. The excess of periodate was destroyed with ethylene glycol (2 mL), and the mixture was stored for 2 h at room temperature, then dialysed against running tap water (48 h) and bidistilled water (24 h, 5 × 5 L), and lyophilised. To a solution of the oxidised polysaccharide in bidistilled water (10 mL) was added $NaBH_4$ (150 mg), the mixture was stored for 18 h at ambient temperature, the excess of $NaBH_4$ was destroyed by the addition of Dowex 50W-X8 (H^+) resin (Bio-Rad), and boric acid was removed by co-concentration with MeOH under reduced pressure.

A solution of an aliquot (10 mg) of the oxidised and reduced polysaccharide in aq 90% formic acid (5 mL) was kept for 1 h at 40°, then co-concentrated with bidistilled water, and lyophilised. A solution of the residue in water (10 mL) was treated (18 h, ambient temperature) with $NaBD_4$ (60 mg). The excess of $NaBD_4$ was removed as described above. The final preparation was eluted from a column (36 × 1.5 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) with bidistilled water at 6.5 mL/h (0.85-mL fractions) and refractive-index monitoring of the eluate. The major fraction was rechromatographed on a second column (95 × 1.2 cm) of Bio-Gel P-2 (4.5 mL/h, 1-mL fractions).

Partial acid hydrolysis.—A solution of the polysaccharide (40 mg) in 0.5 M trifluoroacetic acid (10 mL) was kept for 24 h at 60°, then lyophilised, and the residue was eluted from a column (98 × 1.4 cm) of Bio-Gel P-4 (200–400 mesh) with bidistilled water at 10.4 mL/h (2-mL fractions) and refractive-index monitoring.

High-performance anion-exchange chromatography with pulsed amperometric detection.—Oligosaccharide fractions were subjected to HPAEC-PAD on a Dionex LC system, consisting of a Dionex Bio-LC quaternary gradient module, a model PAD-2 detector, and a CarboPac PA-1 pellicular anion-exchange column (250 × 9 mm). The elution programs had the following ratios of eluents *A* (0.1 M NaOH) and *B* (0.1 M NaOH containing M NaOAc) at ambient temperature and 5 mL/min: *1*, 100:0 for 0.3 min then to 50:50 in 60 min; *2*, 99:1 for 0.3 min then to 50:50 in 60 min; *3*, 95:5 for 0.3 min then to 85:15 in 30 min; *4*, 93:7 for 0.3 min then to 83:17 in 30 min. The PAD involved a gold working-electrode and triple-pulse amperometry, comprising the following pulse potentials and durations: E_1 0.05 V, t_1 300 ms; E_2 0.65 V, t_2 60 ms; E_3 −0.95 V, t_3 180 ms; response time, 1 s. Data were collected and plotted with a Shimadzu C-R3a integrator. Fractions were neutralised immediately with M HCl, lyophilised, and desalted on a column (95 × 1.2 cm) of Bio-Gel P-2 by elution with bidistilled water and refractive-index monitoring. Residual NaOAc was removed on a column (6 × 0.5 cm) of Dowex

50W-X8 (H^+) resin (100–200 mesh, Bio-Rad) by elution with bidistilled water, and acetic acid was removed by lyophilisation.

TLC.—Silica Gel 60 F_{254} (Merck) and 3:2:2 1-butanol–EtOH–H_2O were used, and detection was with orcinol–H_2SO_4.

FABMS.—Positive-ion FAB-mass spectra were recorded with a VG Analytical ZAB-HF mass spectrometer (Xe beam, 7.6 keV; acceleration voltage, 8 kV) with glycerol as the matrix. Linear mass scans over 1500 Da were recorded with a UV chart recorder (Department of Mass Spectrometry).

NMR spectroscopy.—Proton-decoupled ^{13}C-NMR spectra (external Me_4Si; internal dioxane, δ 67.40) were recorded with a Bruker WP-200 spectrometer, equipped with a 10-mm broad-band probe, on solutions in D_2O at 70°.

For the ^{1}H-NMR spectra, carbohydrate samples were repeatedly exchanged in D_2O (99.9 atom% D) with intermediate lyophilisation, then dissolved in 0.4 mL of D_2O (99.96 atom% D). Resolution-enhanced 500-MHz spectra (internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate; internal acetone, δ 2.225) were recorded with a Bruker AM-500 spectrometer (Department of NMR Spectroscopy) at 27°.

2D Homonuclear Hartmann–Hahn (HOHAHA) spin-lock experiments were recorded using the pulse sequence 90°–t_1–SL–acq[16–19], where SL stands for a multiple of the MLEV-17 sequence. The spin-lock field strength corresponded to a 90° pulse width of 27 μs. The total spin-lock mixing time ranged from 35 to 100 ms. The spectral width ranged from 1500 to 3000 Hz in each dimension.

2D Rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out using the pulse sequence 90ϕ°–t_1–SL–acq[20], where SL stands for a continuous spin-lock pulse of 200 or 250 ms at a field strength corresponding to a 90° pulse width between 100–110 μs. The carrier frequency was placed at the left side of the spectrum at 5.7 ppm in order to minimise HOHAHA-type magnetisation transfer. The HOD signal was suppressed by presaturation during 1.0 s. The spectral width ranged from 1500 to 3000 Hz in each dimension.

For the HOHAHA spectra, 256 or 512 experiments of 2K data points were recorded, and, for the ROESY spectra, 512 experiments of 2K data points. The time-proportional phase-increment method (TPPI)[21] was used to create t_1-amplitude modulation. Each data matrix was zero-filled to 2K × 4K and multiplied in each time domain with a phase-shifted sine function, shifted $\pi/3$ for the HOHAHA and $\pi/2$ for the ROESY, prior to phase-sensitive FT.

RESULTS AND DISCUSSION

Isolation and composition of the polysaccharide.—Polysaccharide preparations were isolated as acetone precipitates from the culture supernatant solutions of strain H414, grown in a defined medium or skimmed milk, and purified exopolysaccharides were obtained by fractional precipitation of the total acetone precipitate from aqueous solution at 40% acetone in yields of 53% and 50%, respectively. Contaminating protein (1%) was removed by gel filtration chromatog-

TABLE I

Methylation analysis data of (A) the native polysaccharide isolated from a defined medium, (B) the native polysaccharide isolated from skimmed milk, and (C) oligosaccharide **1** obtained by Smith degradation

Derivative	Molar ratio		
	A	B	C [a]
2,3,4,6-Gal [b,c]	0.7	0.8	1.5
2,4,6-Gal	2.3	2.4	
2,3,6-Gal	1.2	1.2	
2,6-Gal	1.0	1.0	1.0

[a] The expected 2-linked threitol was not observed due to its high volatility. [b] 2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol-*1-d*, etc. [c] Because of the relatively high volatility of this residue, the value is lower than expected.

raphy on Sephacryl S-500. Each purified polysaccharide had a carbohydrate content of 99% and contained only D-galactose, as shown by GLC of the trimethylsilylated methyl glycosides and (−)-2-butyl glycosides. The ^{13}C-NMR spectra of the two preparations were identical (data not shown), and C-1 signals were detected in the region 101–106 ppm. The signals at δ 61.86 and 61.20 were assigned to C-6. GLC of the partially methylated alditol acetates, obtained from the methylated polysaccharides, revealed, for each preparation, terminal, 3-linked, 4-(or 5-)linked, and 3,4-(or 3,5-)linked Gal residues (Table I), suggesting a branched galactan with a pentasaccharide repeating unit. In view of the absence of 6-linked residues, the ^{13}C-NMR data for C-6 indicated[22] all Gal residues to be pyranoid (α-D-Gal*p*-OMe δ 62.2, β-D-Gal*p*-OMe δ 62.0, α-D-Gal*f*-OMe δ 64.1, β-D-Gal*f*-OMe δ 63.6). The ^{1}H-NMR spectrum at 70° contained signals for H-1 at δ 5.023 (bs, residue **A**), 4.90, 4.72, and 4.69 (3 bd, $J_{1,2}$ 7–8 Hz, residues **B**, **D**, and **C**, respectively), and 4.642 (d, $J_{1,2}$ 7.1 Hz, residue **E**) in nearly equimolar ratios, indicative of a pentasaccharide repeating unit.

Smith degradation.—The polysaccharide, isolated from the defined medium, was oxidised with periodate, reduced with borohydride, subjected to mild hydrolysis with acid, and reduced with borodeuteride. The degraded material was fractionated on Bio-Gel P-2. Rechromatography of the major fraction on Bio-Gel P-2 yielded two major fractions and one minor fraction (I–III, respectively).

Fraction II gave only one peak on CarboPac PA-1 (Fig. 1*A*). The molecular mass of the oligosaccharide **1**, as determined by FABMS, was 608 (Hex_3-tetritol). Monosaccharide analysis indicated the presence of Gal and threitol in the molar ratio 3.0:1.1, and methylation analysis revealed terminal and 3,4-disubstituted Gal (Table I). The ^{1}H-NMR spectrum (Fig. 2) contained signals at δ 5.123 (bs, residue **A**, H-1α), 4.828 (d, $J_{1,2}$ 7.9 Hz, residue **B**, H-1β), and 4.604 (d, $J_{1,2}$ 7.6 Hz, residue **C**, H-1β). The assignments for H-2,3,4 of residues **B** and **C**, and for H-2,3,4,5,6a,6b of residue **A**, were made on the basis of cross-peaks observed in the HOHAHA spectrum (not shown), and the NMR data are listed in Table II. In the ROESY

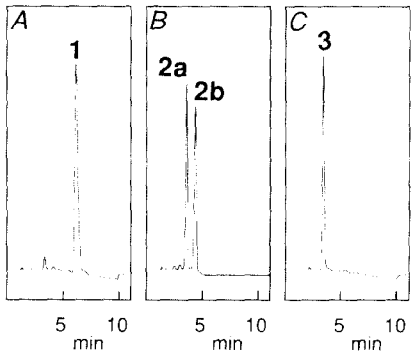


Fig. 1. HPAEC-PAD fractionation patterns (program *1*) on CarboPac PA-1 of Bio-Gel P-2 fractions II (*A*), I (*B*), and III (*C*), obtained by Smith degradation of the polysaccharide.

spectrum (not shown), the inter-residue NOEs between **B** H-1 and **A** H-4 and between **C** H-1 and **A** H-3 or **A** H-2 indicated the presence of a 3,4- or 2,4-disubstituted α-Gal residue **A**. Furthermore, an inter-residue NOE was observed for **A** H-1 with a proton resonating at δ 3.79, which was identified as threitol H-2 based on the fact that the threitol residue must stem from the 4-linked Gal in the

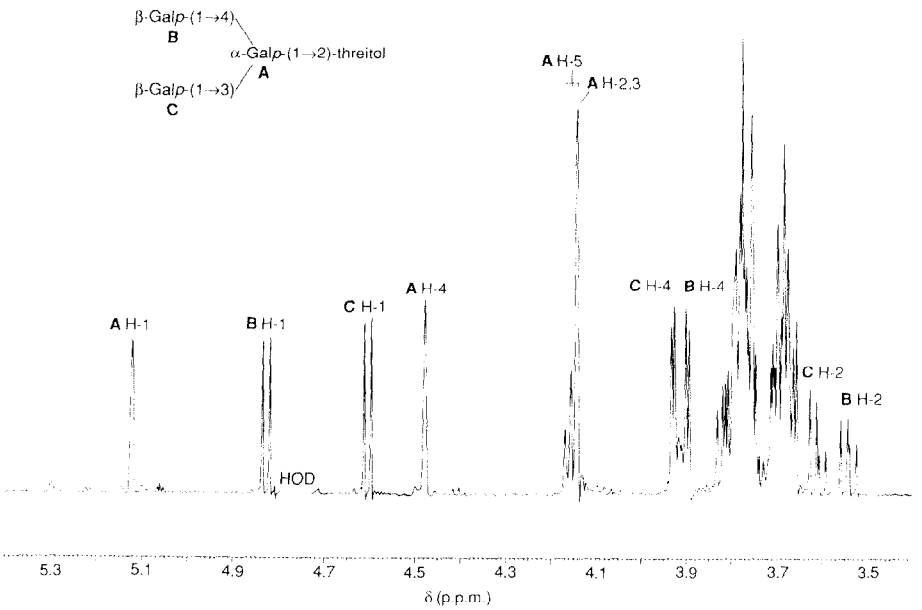


Fig. 2. 500-MHz ^{1}H-NMR spectrum of oligosaccharide **1**.

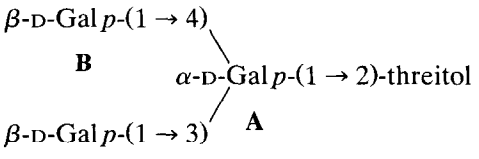
TABLE II

^{1}H-NMR chemical shifts [a] of **1** and **3**, obtained by Smith degradation of the native polysaccharide

Residue	Proton	**1**	**3** [b]
A(-ol)	H-1	5.123	n.d.
	H-2	4.142	4.176
	H-3	4.142	4.088
	H-4	4.478	4.088
	H-5	4.155	4.227
	H-6a [c]	3.70	3.690
	H-6b	3.82	3.823
B	H-1	4.828	4.531
	H-2	3.541	3.545
	H-3	3.69	3.669
	H-4	3.897	3.904
C	H-1	4.604	4.460
	H-2	3.609	3.580
	H-3	3.67	3.671
	H-4	3.929	3.898

[a] In ppm relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ 2.225) in D_2O at 27°. [b] Residues **B** and **C** in **3** can be interchanged. [c] Chemical shifts of H-6a and H-6b can be interchanged.

native polysaccharide. The combined results lead to the structure **1**.

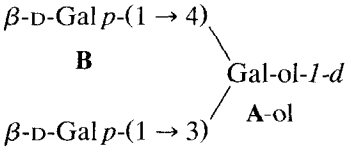


1

Fraction I was separated into the oligosaccharides **2a** and **2b** (Fig. 1*B*) each with a molecular mass of 650 Da (FABMS). The ^{1}H-NMR spectrum of **2a** contained H-1 signals at δ 5.299 (bs), 4.827 ($J_{1,2}$ 7.6 Hz), and 4.605 ($J_{1,2}$ 7.7 Hz), and a triplet at δ 5.060, and that of **2b** contained doublets at δ 5.093 ($J_{1,2}$ 4.0 Hz), 4.834 ($J_{1,2}$ 7.9 Hz), and 4.626 ($J_{1,2}$ 7.6 Hz), and a triplet at δ 4.891. These results suggested **2a** and **2b** to be cyclic *O*-2′-hydroxyethylidene derivatives of **1**, formed from the polyalcohol of the degraded 4-linked residue during mild acid hydrolysis[23]. Prolonged hydrolysis of fraction I at 70° converted **2a** and **2b** into **1**.

Fraction III gave only one peak on CarboPac PA-1 (Fig. 1*C*), the FABMS spectrum of which contained a peak at m/z 508 for $[M + H]^+$ that corresponded to a trisaccharide-alditol-*1-d* (**3**). The ^{1}H-NMR spectrum revealed two β-Gal residues with H-1 signals at δ 4.531 (d, $J_{1,2}$ 7.8 Hz, residue **B**) and 4.460 (d, $J_{1,2}$ 7.8 Hz, residue **C**). The H-2,3,4 signals of the two Gal residues were assigned from the cross-peaks observed on the H-1 tracks in the HOHAHA spectrum (Table II). The

signals at δ 4.227 (t) and 4.176 (d) were assigned to H-5 and H-2 of Gal-ol-*1-d* (**A**-ol), respectively. On the H-2 and H-5 tracks of the **A**-ol residue in the HOHAHA and ROESY spectra, cross-peaks were observed with H-3,4,6a,6b. In the ROESY spectrum, the inter-residue connectivities **B** H-1,**A**-ol H-4/3, **B** H-1,**A**-ol H-2, and **C** H-1,**A**-ol H-3/4 suggested **A**-ol to be 2,3-, 2,4-, or 3,4-di-substituted. Because 2,3- or 2,4-disubstituted Gal was not present in the native polysaccharide, the Gal-ol-*1-d* must be 3,4-disubstituted. Therefore the structure of **3** is as shown.

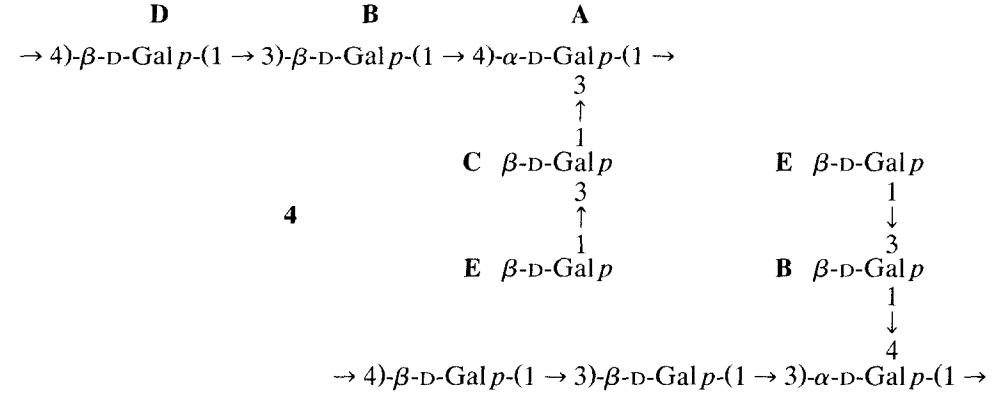


3

Like **2a** and **2b**, **3** was a by-product, and was formed during partial hydrolysis from **1** by cleavage of the Gal → threitol linkage.

Smith degradation of the exopolysaccharide isolated from bacteria grown in skimmed milk gave the same results as those noted above (data not shown).

Based on the methylation analysis and ^{1}H-NMR data of the native polysaccharide and the formation of **1** after Smith degradation, the structures **4** and **5** can be proposed for the polysaccharide in which a disaccharide side chain is 3- and 4-linked to α-Gal, respectively.



4

5

Partial acid hydrolysis.—In order to discriminate between structures **4** and **5**, oligosaccharides were prepared by partial acid hydrolysis of the polysaccharide isolated from skimmed milk. Fractionation of the hydrolysate on Bio-Gel P-4 gave 13 fractions (data not shown). Fraction *I* contained only Gal (TLC). Fractions *II–VII* were fractionated on CarboPac PA-1 with program *2* (Fig. 3). Each fraction

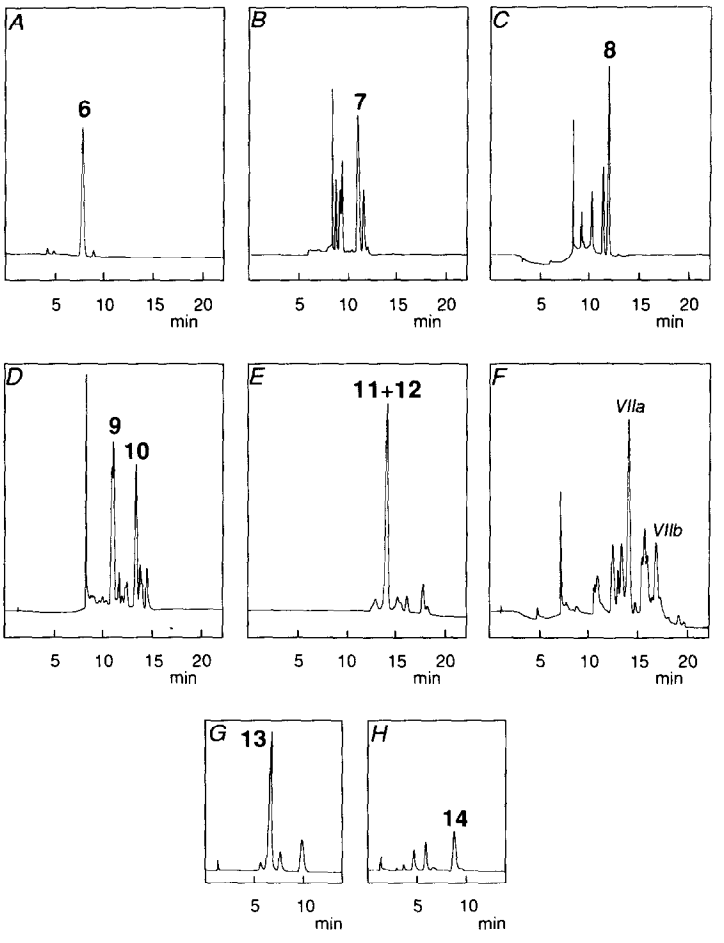


Fig. 3. HPAEC-PAD fractionation patterns on CarboPac PA-1 of Bio-Gel P-4 fractions *II* (*A*), *III* (*B*), *IV* (*C*), *V* (*D*), *VI* (*E*), and *VII* (*F*), and of the HPAEC-PAD fractions *VIIa* (*G*) and *VIIb* (*H*): program *2* for *II*–*VII*, program *3* for *VIIa*, and program *4* for *VIIb*.

afforded one or two major components which were desalted and reduced with $NaBD_4$. Fraction *VII* gave subfractions *VIIa* and *VIIb* which were purified further on CarboPac PA-1 with programs *3* and *4*, respectively, to yield one major component, **13** and **14**, respectively (Fig. 3). In this way, oligosaccharide-alditols **6**–**14** were obtained.

Oligosaccharide-alditol **6**.—In the ^{1}H-NMR spectrum of **6**, the only H-1 signal was at δ 4.444 (d, $J_{1,2}$ 7.7 Hz, H-1β), and there were signals for Gal-ol-*1-d* H-2 (d) and H-5 (t) at δ 4.052 and 4.209, respectively. FABMS showed the molecular mass of methylated **6** to be 471 indicative of a disaccharide-alditol-*1-d*. The presence of a peak at m/z 133 for $(CH_2OMeCHOMeCHOMe)^+$ in the EI-mass spectrum of methylated **6** indicated[24] a (1 → 3) linkage, thereby establishing the structure of **6**.

β-Gal*p*-(1 → 3)-Gal-ol-*1-d* **6**

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)-Gal-ol-*1-d* **7**

E_4/D_5 **C** **A**-ol

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)-α-Gal*p*-(1 → 4)-Gal-ol-*1-d* **8**

E_4/D_5 **C** **A** **D***-ol

β-Gal*p*-(1 → 4)
B \
α-Gal*p*-(1 → 4-Gal-ol-*1-d* **9**
β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)/ **A** **D***-ol

E_4/D_5 **C**

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)-α-Gal*p*-(1 → 4)-β-Gal*p*-(1 → 3)-Gal-ol-*1-d* **10**

E_4/D_5 **C** **A** **D*** **B_4^*/C_5^***-ol

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)
D_4/E_5 **B** \
α-Gal*p*-(1 → 4)-Gal-ol-*1-d* **11**
β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)/ **A** **D***-ol

E_4/D_5 **C**

β-Gal*p*-(1 → 4)
B \
α-Gal*p*-(1 → 4)-β-Gal*p*-(1 → 3)-Gal-ol-*1-d* **12**
β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)/ **A** **D*** **B_4^*/C_5^***-ol

E_4/D_5 **C**

β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)
D_4/E_5 **B** \
α-Gal*p*-(1 → 4)-β-Gal*p*-(1 → 3)-Gal-ol-*1-d* **13**
β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)/ **A** **D*** **B_4^*/C_5^***-ol

E_4/D_5 **C**

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

-β-Gal*p*-(1 → 4)-α-Gal*p*-(1 → 4)-Gal-ol-*1-d* **14**
B **A** **D***-ol

Oligosaccharide-alditol **7**.—GLC–(EI)MS indicated that methylated **7** had a molecular mass of 675 [*m/z* 630 ($M^+ - CH_2OMe$), 586 ($M^+ - CH_2OMeCHOMe$)] and the sequence Hex-Hex-Hex-ol-*1-d* followed[25] from the fragment ions with

TABLE III

Methylation analysis data of oligosaccharide-alditols-*1-d* obtained by partial acid hydrolysis/reduction ($NaBD_4$)

Derivative	Molar ratios				
	7	**8**	**9**	**10**	**11+12**
1,2,4,5,6-Gal [a,b]	+			0.4	+
1,2,3,5,6-Gal [b]		+	+		+
2,3,4,6-Gal [c]	0.4	0.1	1.2	0.3	1.4
2,4,6-Gal	1.0	2.0	1.0	2.0	3.0
2,3,6-Gal				1.3	1.6
2,6-Gal			1.0		2.0

[a] 1,2,4,5,6-Gal = 3-mono-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol-*1-d*, etc. [b] Because of the relatively high volatility of these residues and the undermethylation resulting mainly in 1,2,4,6-Gal for 1,2,4,5,6-Gal and 1,3,5,6-Gal for 1,2,3,5,6-Gal, the values are lower than expected. [c] Because of the relatively high volatility of this residue, the value is lower than expected.

m/z 219 (aA_1), 423 (baA_1), 440 (bcA_1), and 236 (cA_1). The linkage between Hex and Hex-ol-*1-d* was established to be (1 → 3), because of the presence of the fragment ions *m/z* 133 $(CH_2OMeCHOMeCHOMe)^+$, 586 $(M^+ - CH_2OMeCHOMe)$, and 542 $(M^+ - CH_2OMeCHOMeCHOMe)$[25]. The linkage between Hex and Hex was also assigned as (1 → 3), indicated by an intense peak at *m/z* 159 (composed probably of fragments of the internal Hex residue[25]), and further supported by the methylation analysis data of methylated **7** (Table III).

The ^{1}H-NMR spectrum of **7** contained two H-1β signals of equal intensity at δ 4.613 (d, $J_{1,2}$ 7.6 Hz, residue $\mathbf{E_4}$ or $\mathbf{D_5}$, wherein $\mathbf{E_4}$ means residue **E** in repeating unit **4** and $\mathbf{D_5}$ residue **D** in repeating unit **5**) and 4.505 (d, $J_{1,2}$ 7.8 Hz, residue **C**). On the H-1 tracks of each Gal in the HOHAHA spectrum, cross-peaks were detected with H-2,3,4. The H-5 signals of each residue were assigned from their connectivities with H-4 in the ROESY spectrum. The signals for Gal-ol-*1-d* (**A**-ol) H-5 (δ 4.221, t) and H-2 (δ 4.056, d) were used as starting points for the assignments of the other ^{1}H resonances of the **A**-ol residue on the basis of cross-peaks observed on the H-5 and H-2 tracks in the HOHAHA and ROESY spectra. The chemical shifts are listed in Table IV. In the ROESY spectrum, $\mathbf{E_4}/\mathbf{D_5}$ H-1 showed inter-residue NOEs with **C** H-3 (strong) and **C** H-2,4 (weak), in accordance with a (1 → 3) linkage. For **C** H-1, an inter-residue NOE was observed with H-3 of **A**-ol, establishing the linkage between **C** and **A**-ol as (1 → 3) and confirming the structure **7**.

Oligosaccharide-alditol **8**.—Methylation analysis of **8** revealed terminal and 3-linked Gal, and 4-linked Gal-ol-*1-d* (Table III). The ^{1}H-NMR spectrum contained three H-1 signals of equal intensity at δ 5.139 (d, $J_{1,2}$ 3.7 Hz), 4.678 (d, $J_{1,2}$ 7.7 Hz), and 4.621 (d, $J_{1,2}$ 7.6 Hz), indicating a tetrasaccharide-alditol with one α- and two β-Gal residues. Based on the ^{1}H-NMR data of other oligosaccharide-alditols (**9**, **11**, and **14**), these signals were assigned to residue **A** (1 → 4)-linked to

TABLE IV

^{1}H-NMR chemical shifts [a] of oligosaccharide-alditols obtained by partial acid hydrolysis/reduction ($NaBD_4$)

Residue	Proton	Oligosaccharide-alditol							
		7	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A	H-1		5.139	5.146	4.969	5.147	4.956	4.959	5.124
	H-2			4.16 [b]	4.022	4.17	4.16	4.166 [b]	3.944
	H-3			4.16 [b]	4.090	4.17	4.16	4.166 [b]	4.002
	H-4			4.492	4.302	4.51	4.51	4.519	4.275
	H-5			4.133	4.387			4.435	4.124
	H-6a [c]			3.70 [b]	3.70 [b]			3.631 [b]	
	H-6b			3.83 [b]				3.807 [b]	
B	H-1			4.832		4.899	4.846	4.913	4.653
	H-2			3.540		3.70	3.553	3.716 [b]	3.770 [d]
	H-3			3.693 [b]		3.87	3.70	3.878	3.890 [d]
	H-4			3.89 [b]		4.17	3.90	4.180 [b]	4.149
C	H-1	4.505	4.678	4.671	4.682	4.678 [c]	4.669 [c]	4.678	
	H-2	3.737 [b]		3.78 [b]	3.770 [b]	3.78	3.78	3.786 [b]	
	H-3	3.839 [b]		3.844 [b]	3.850 [b]	3.85	3.85	3.860	
	H-4	4.184 [b]		4.202	4.202	4.204	4.204	4.208	
	H-5	3.79 [b]			3.721 [b]			3.72 [b]	
D	H-1								4.700
	H-2								3.67 [d]
	H-3								3.766 [d]
	H-4								4.053
D_4 [f]	H-1					4.63		4.627	
	H-2					3.615		3.61	
	H-3					3.672		3.676 [b]	
	H-4					3.925		3.921 [b]	
E_4 [g]	H-1	4.613	4.621	4.622	4.624	4.63	4.63	4.632	
	H-2	3.604 [b]		3.612	3.609	3.615	3.615	3.609	
	H-3	3.665 [b]		3.668 [b]	3.669 [b]	3.672	3.672	3.676 [b]	
	H-4	3.921 [b]		3.924	3.924	3.925	3.925	3.921 [b]	
	H-5	3.690 [b]			3.69 [b]				
***A**	H-1								4.995
	H-2								4.023
	H-3								4.10 [d]
	H-4								4.304
	H-5								4.409
	H-6a/6b								3.71 [d]
***C**	H-1								4.677
	H-2								3.78 [d]
	H-3								3.86 [d]
	H-4								4.204
***E**	H-1								4.623
	H-2								3.61 [d]
	H-3								3.68 [d]
	H-4								3.925

TABLE IV (continued)

Residue	Proton	Oligosaccharide-alditol							
		7	8	9	10	11	12	13	14
D*	H-1				4.510		4.510	4.511	
	H-2				3.642 [b]		3.645	3.654 [b]	
	H-3				3.75 [b]		3.757	3.767 [b]	
	H-4				4.040		4.025	4.027	
	H-5				3.785 [b]			3.793 [b]	
	H-6a				3.85 [b]			3.85 [b]	
	H-6b				3.961 [b]			3.96 [b]	
A-ol	H-1	3.775 [b]							
	H-2	4.056 [b]							
	H-3	3.925 [b]							
	H-4	3.802 [b]							
	H-5	4.221 [b]							
	H-6a/6b	3.69 [b]							
B$_4^*$-ol [h]	H-1				3.785 [b]			3.79 [b]	
	H-2				4.067			4.067	
	H-3				3.932 [b]			3.934 [b]	
	H-4				3.813 [b]			3.82 [b]	
	H-5				4.166			4.18 [b]	
	H-6a/6b				3.685 [b]			3.69 [b]	
D*-ol	H-4			3.89 [b]					3.89 [d]
	H-5			4.095					4.092
	H-6a/6b			3.80 [b]					3.80 [d]

[a] In ppm relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ 2.225) in D_2O at 27°, unless otherwise indicated. [b,d] At 8 and 5°, respectively. [c] Chemical shifts of H-6a and H-6b can be interchanged. [e] Chemical shifts can be interchanged. [f] Residue **D**$_4$ or **E**$_5$. [g] Residue **E**$_4$ or **D**$_5$. [h] Residue **B**$_4^*$-ol or **C**$_5^*$-ol.

D*-ol, residue **C** (1 → 3)-linked to residue **A**, and the terminal Gal residue **E**$_4$/**D**$_5$ (1 → 3)-linked to residue **C**, respectively, giving the structure of **8**.

Oligosaccharide-alditol **9**.—Methylation analysis of **9** revealed terminal, 3-, and 3,4-linked Gal, and 4-linked Gal-ol-*1-d* (Table III), indicating a branched structure. The ^{1}H-NMR spectrum contained four H-1 signals of equal intensity at δ 5.146 (d, $J_{1,2}$ 3.3 Hz, residue **A**), 4.832 (d, $J_{1,2}$ 7.9 Hz, residue **B**), 4.671 (d, $J_{1,2}$ 7.6 Hz, residue **C**), and 4.622 (d, $J_{1,2}$ 7.6 Hz, residue **E**$_4$/**D**$_5$) suggesting a pentasaccharide-alditol with one α- and three β-Gal residues. On the various H-1 tracks of the Gal residues in the HOHAHA spectrum, cross-peaks with H-2,3,4 were observed, whereas H-5,6a,6b of residue A were found via the H-4,5 tracks. A signal at δ 4.095 (bt) was assigned to H-5 of the Gal-ol-*1-d* residue (**D***-ol), and this proton showed cross-peaks with H-6a and/or H-6b in the HOHAHA spectrum and with H-4 in the ROESY spectrum. The chemical shifts are given in Table IV. The ROESY spectrum showed inter-residue NOEs for **E**$_4$/**D**$_5$ H-1,**C** H-3, **B** H-1,**A** H-4, **C** H-1,**A** H-3 and/or **A** H-2, and **A** H-1,**D***-ol H-4, yielding evidence for the sequence of the Gal residues including the linkage positions. A (1 → 2) linkage

between the **C** and **A** residues can be excluded on the basis of the results of the methylation analysis (Table III). Based on the various data, the structure of **9** is assigned.

Oligosaccharide-alditol **10**.—Methylation analysis of **10** revealed terminal, 3-, and 4-linked Gal, and 3-linked Gal-ol-*1-d* (Table III), indicating a linear structure. The ^{1}H-NMR spectrum contained four H-1 signals of equal intensity at δ 4.969 (d, $J_{1,2}$ 4.0 Hz, residue **A**), 4.682 (d, $J_{1,2}$ 7.8 Hz, residue **C**), 4.624 (d, $J_{1,2}$ 7.6 Hz, residue $\mathbf{E_4/D_5}$), and 4.510 (d, $J_{1,2}$ 7.3 Hz, residue **D***), suggesting a pentasaccharide-alditol with one α- and three β-Gal residues. The assignments of the non-anomeric protons of these residues (Table IV) are based on cross-peaks observed in the HOHAHA and ROESY spectra. The signals at δ 4.166 (t) and 4.067 (d), assigned to H-5 and H-2, respectively, of the Gal-ol-*1-d* residue ($\mathbf{B_4^*/C_5^*}$-ol) were used as starting points for the determination of the other protons of the $\mathbf{B_4^*/C_5^*}$-ol residue via cross-peaks observed on the H-5 and H-2 tracks in the HOHAHA and ROESY spectra (Table IV). The sequence $\mathbf{E_4/D_5 \rightarrow C \rightarrow A \rightarrow D^* \rightarrow B_4^*/C_5^*}$-ol and the linkage positions followed from inter-residue NOEs observed in the ROESY spectrum. On basis of the connectivities $\mathbf{E_4/D_5}$ H-1,**C** H-3 (strong), H-2,4 (weak) and **D*** H-1,$\mathbf{B_4^*/C_5^*}$-ol H-3 (strong), H-4 (weak), (1 → 3) linkages were established. For the **C** → **A** linkage, two connectivities were observed, namely, **C** H-1,**A** H-2,3, but a **C**-(1 → 2)-**A** linkage can be excluded on the basis of the methylation analysis data (Table III). For the **A** → **D*** linkage, a NOE was observed between **A** H-1 and **D*** H-4, but, in view of the observation that an α-GalNAc-(1 → 3)-Gal linkage gives rise to a strong NOE between H-1 of α-GalNAc and H-4 of Gal[26–28], the linkage between **A** and **D*** cannot be assigned unambiguously as (1 → 4). However, because methylation analysis revealed a 4-linked residue (Table III), and the other linkages are all (1 → 3), the **A** → **D*** linkage was established as (1 → 4). The final structure **10** is assigned.

Oligosaccharide-alditols **11** *and* **12**.—HPAEC-PAD of Bio-Gel P-4 fraction *VI* gave one major subfraction. After reduction with $NaBD_4$, methylation analysis of this subfraction revealed terminal, 3-, 4-, and 3,4-linked Gal, and 3- and 4-linked Gal-ol-*1-d*, indicating a mixture of **11** and **12**. Because **11** and **12** could not be separated by HPAEC, the mixture as such was analysed. FABMS gave a molecular mass of 993 Da, corresponding to a hexasaccharide-alditol-*1-d*. The ^{1}H-NMR spectrum contained ten H-1 signals of nearly equal intensity at δ 5.147 [d, $J_{1,2}$ 3.5 Hz, residue **A(11)**], 4.956 [d, $J_{1,2}$ 2.9 Hz, residue **A(12)**], 4.899 [d, $J_{1,2}$ 8.0 Hz, residue **B(11)**], 4.846 [d, $J_{1,2}$ 7.9 Hz, residue **B(12)**], 4.678 [d, $J_{1,2}$ 7.6 Hz, residue **C(11)**], 4.669 [d, $J_{1,2}$ 7.7 Hz, residue **C(12)**], 4.63 [3 overlapping signals, $J_{1,2}$ 7.8 Hz, residues $\mathbf{D_4/E_5(11)}$, $\mathbf{E_4/D_5(11)}$, $\mathbf{E_4/D_5(12)}$], and 4.510 [d, $J_{1,2}$ 7.6 Hz, residue **D*****(12)**], suggesting the presence of two hexasaccharide-alditols in the molar ratio 1:1. The H-2,3,4 signals of all of the Gal residues were assigned from the cross-peaks observed on the H-1 tracks in the HOHAHA spectrum, and the data are listed in Table IV. No assignments could be made for the Gal-ol-*1-d* residues **D*****(11)**-ol and $\mathbf{B_4^*/C_5^*(12)}$-ol. Taking into account the methylation analysis data

(Table III) and the molecular masses, the sequence of the residues $\mathbf{E_4/D_5} \rightarrow \mathbf{C} \rightarrow [\mathbf{D_4/E_5} \rightarrow \mathbf{B} \rightarrow]\mathbf{A} \rightarrow \mathbf{D}^*$-ol for **11** and $\mathbf{E_4/D_5} \rightarrow \mathbf{C} \rightarrow [\mathbf{B} \rightarrow]\mathbf{A} \rightarrow \mathbf{D}^* \rightarrow \mathbf{B_4^*/C_5^*}$-ol for **12**, together with the linkage positions, could be deduced from the inter-residue NOEs observed in the ROESY spectrum. For **11**, the $\mathbf{E_4/D_5}$-(1 → 3)-**C**, **C**-(1 → 3)-**A**, $\mathbf{D_4/E_5}$-(1 → 3)-**B**, and **B**-(1 → 4)-**A** elements were deduced from the detection of the connectivities $\mathbf{E_4/D_5}$ H-1,**C** H-3, **C** H-1,**A** H-3 (and/or H-2), $\mathbf{D_4/E_5}$ H-1,**B** H-3, and **B** H-1,**A** H-4, respectively. For **A** H-1, a connectivity was observed with a proton resonating at δ 3.89 identified as **D***-ol H-4, based on the ^{1}H-NMR data for **9** (Table IV). Thus, the structure is suggested to be **11**. For **12**, the NOEs $\mathbf{E_4/D_5}$ H-1,**C** H-3, **C** H-1,**A** H-3 (and/or H-2), **B** H-1,**A** H-4, and **A** H-1,**D*** H-4 yielded the elements $\mathbf{E_4/D_5}$-(1 → 3)-**C**, **C**-(1 → 3)-**A**, **B**-(1 → 4)-**A**, and **A**-(1 → 4)-**D***. The linkage **D***-(1 → 3)-$\mathbf{B_4^*/C_5^*}$-ol was deduced from the connectivity between **D*** H-1 and a proton resonating at δ 3.93, which was identified as $\mathbf{B_4^*/C_5^*}$-ol H-3 on the basis of the ^{1}H-NMR data for **10** (Table IV), and the structure **12** is suggested.

Oligosaccharide-alditol **13**.—The ^{1}H-NMR spectrum of **13** contained six H-1 signals of equal intensity at δ 4.959 (d, $J_{1,2}$ 3.0 Hz, residue **A**), 4.913 (d, $J_{1,2}$ 8.0 Hz,

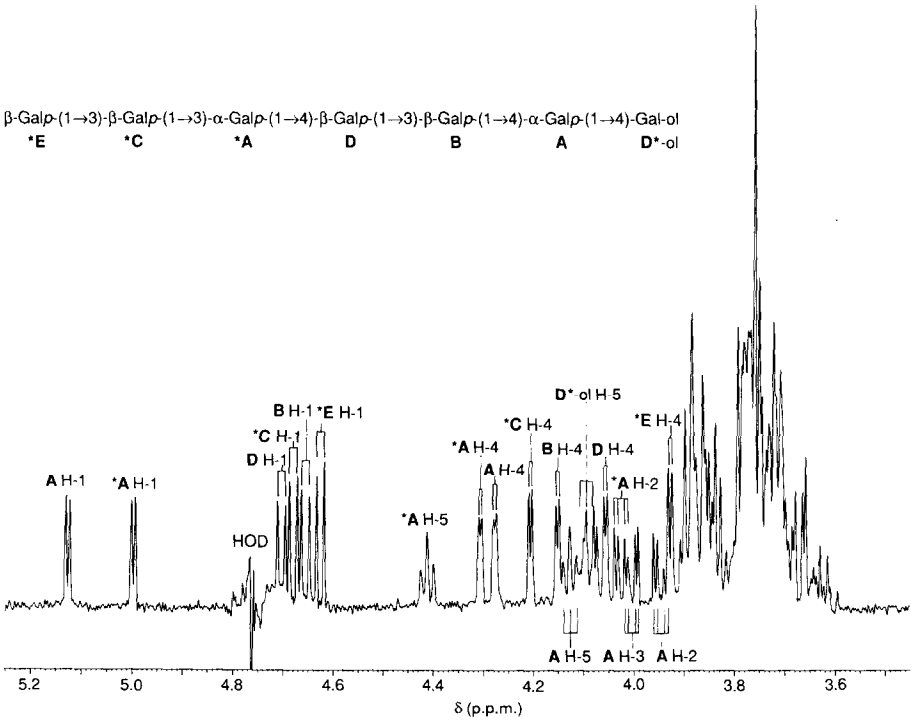


Fig. 4. 500-MHz ^{1}H-NMR spectrum of oligosaccharide-alditol **14** recorded at 27°.

residue **B**), 4.678 (d, $J_{1,2}$ 7.7 Hz, residue **C**), 4.632 (d, $J_{1,2}$ 7.7 Hz, residue $\mathbf{E_4/D_5}$), 4.627 (d, $J_{1,2}$ 7.6 Hz, residue $\mathbf{D_4/E_5}$), and 4.511 (d, $J_{1,2}$ 7.9 Hz, residue **D***), suggesting a heptasaccharide-alditol with one α- and five β-Gal residues. The assignments of the non-anomeric protons of these residues (Table IV) are based on cross-peaks observed in the HOHAHA and ROESY spectra. The signal at δ 4.067 (d), assigned to H-2 of the $\mathbf{B_4^*/C_5^*}$-ol residue, was used as the starting point for the determination of the other protons of $\mathbf{B_4^*/C_5^*}$-ol via cross-peaks observed in the HOHAHA and ROESY spectra (Table IV). Taking into account the methylation analysis data of the native polysaccharide (Table I), the sequence $\mathbf{E_4/D_5 \rightarrow C \rightarrow [D_4/E_5 \rightarrow B \rightarrow]A \rightarrow D^* \rightarrow B_4^*/C_5^*}$-ol and the linkage positions followed from inter-residue NOEs observed in the ROESY spectrum. On the basis of the connectivities $\mathbf{E_4/D_5}$ H-1,**C** H-3, **C** H-1,**A** H-3 (and/or H-2), $\mathbf{D_4/E_5}$ H-1,**B** H-3, and **D*** H-1,$\mathbf{B_4^*/C_5^*}$-ol H-3, the linkages for these elements were established

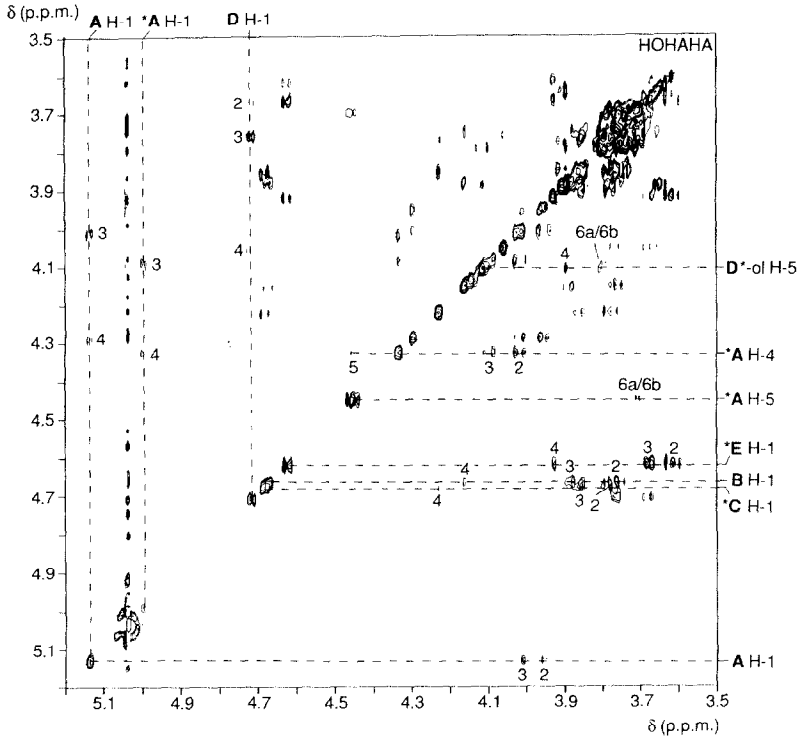


Fig. 5. 500-MHz 2D HOHAHA spectrum of oligosaccharide-alditol **14** recorded at 5° with a mixing time of 100 ms. Diagonal peaks of H-1 of the various residues, of H-4,5 of residue ***A**, and of H-5 of residue **D***-ol are indicated. The numbers near the cross-peaks refer to the protons of the scalar-coupling network belonging to a diagonal peak.

as (1 → 3). The (1 → 4) linkages between **B** and **A** and between **A** and **D*** were deduced from the **B** H-1,**A** H-4 and **A** H-1,**D*** H-4 cross-peaks, respectively. The structure **13** is assigned.

Oligosaccharide-alditol **14**.—The ^{1}H-NMR spectrum of **14** (Fig. 4) contained six H-1 signals of equal intensity at δ 5.124 (d, $J_{1,2}$ 3.9 Hz, residue **A**), 4.995 (d, $J_{1,2}$ 3.9 Hz, residue ***A**), 4.700 (d, $J_{1,2}$ 7.7 Hz, residue **D**), 4.677 (d, $J_{1,2}$ 7.8 Hz, residue ***C**), 4.653 (d, $J_{1,2}$ 7.9 Hz, residue **B**), and 4.623 (d, $J_{1,2}$ 7.6 Hz, residue ***E**), suggesting a heptasaccharide-alditol with two α- and four β-Gal residues. The H-2,3,4 assignments (Table IV) of these residues (except ***A**) were made on basis of the cross-peaks on the H-1 tracks in the HOHAHA spectrum (Fig. 5). For residue ***A**, H-1,2,3,4,5 connectivities were found on the ***A** H-4 track, whereas H-6a and/or H-6b was detected on the *A H-5 track. The H-5 atom of residue **A** was identified on the basis of a cross-peak on the **A** H-4 track in the ROESY spectrum (Fig. 6).

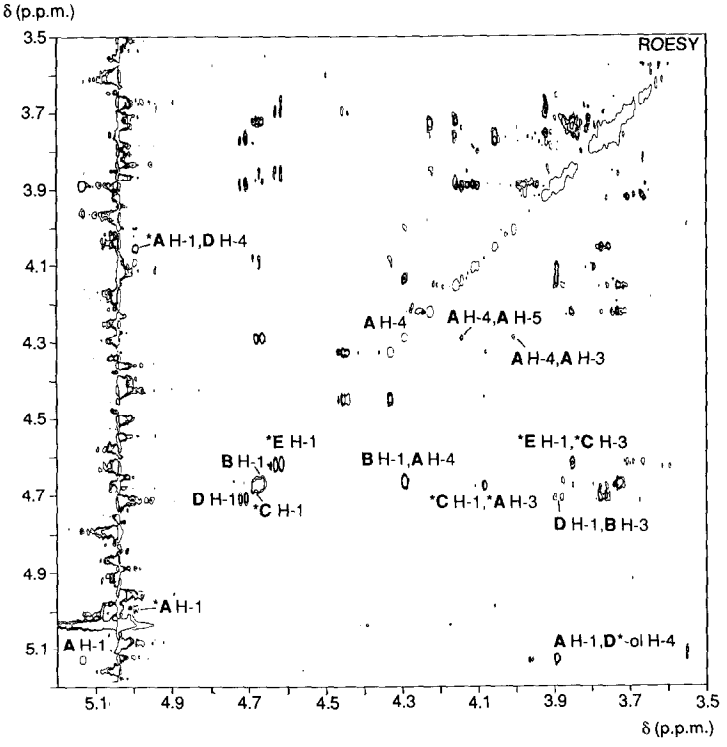
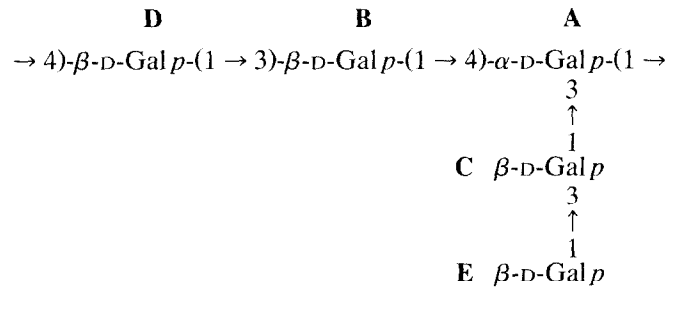


Fig. 6. 500-MHz 2D ROESY spectrum of oligosaccharide-alditol **14** recorded at 5° with a mixing time of 200 ms. Only inter-residue NOEs along the H-1 tracks and the intra-residue NOEs along the **A** H-4 track are given. **A** H-1,**D***-ol H-4 means the cross-peak between H-1 of residue **A** and H-4 of residue **D***-ol, etc.

The H-5 signal of the Gal-ol-*1-d* residue (**D***-ol) at δ 4.092 was used as starting point for the determination of H-4 and H-6a and/or H-6b via cross-peaks observed in the HOHAHA spectrum. The chemical shifts are compiled in Table IV. The inter-residue connectivities ***E** H-1,***C** H-3, ***C** H-1,***A** H-3, ***A** H-1,**D** H-4, **D** H-1,**B** H-3,**B** H-1,**A** H-4, and **A** H-1,**D***-ol H-4 observed on the H-1 tracks in the ROESY spectrum (Fig. 6) established the structure of **14** as ***E**-(1 → 3)-***C**-(1 → 3)-***A**-(1 → 4)-**D**-(1 → 3)-**B**-(1 → 4)-**A**-(1 → 4)-**D***-ol.

In summary, the finding of oligosaccharide-alditol **14** excludes proposal **5** for the structure of the polysaccharide, affording **4** as the structure of the exopolysaccharide of *L. lactis* subsp. *cremoris* H414.



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REFERENCES

1 J. Cerning, *FEMS Microbiol. Rev.*, 87 (1990) 113–130.
2 T. Doco, J-M. Wieruszeski, B. Fournet, D. Carcano, P. Ramos, and A. Loones, *Carbohydr. Res.*, 196 (1990) 313–321.
3 M. Oda, H. Hasegawa, S. Komatsu, M. Kambe, and F. Tsuchiya, *Agric. Biol. Chem.*, 47 (1983) 1623–1625.
4 J. Cerning, C. Bouillanne, M.J. Desmazeaud, and M. Landon, *Biotechnol. Lett.*, 10 (1988) 255–260.
5 S.M. Schellhaass, Ph.D. Thesis, University of Minnesota, U.S.A. 1983.

6 D. Macura and P.M. Townsley, *J. Dairy Sci.*, 67 (1984) 735–744.
7 H. Nakajima, S. Toyoda, T. Toba, T. Itoh, T. Mukai, H. Kitazawa, and S. Adachi, *J. Dairy Sci.*, 73 (1990) 1472–1477.
8 R. Otto, B. ten Brink, H. Veldkamp, and W.N. Konings, *FEMS Microbiol. Lett.*, 16 (1983) 69–74.
9 T.D. Thomas, D.C. Ellwood, and V.M. Longyear, *J. Bacteriol.*, 138 (1979) 109–117.
10 J.P. Kamerling and J.F.G. Vliegenthart, in A.M. Lawson (Ed.), *Clinical Biochemistry — Principles, Methods, Applications. Vol. 1, Mass Spectrometry*, Walter de Gruyter, Berlin, 1989, pp. 176–263.
11 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
12 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.
13 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1965) 350–356.
14 A.L. Kvernheim, *Acta Chem. Scand., Ser. B*, 41 (1987) 150–152.
15 P.J. Harris, R.J. Henry, A.B. Blakeney, and B.A. Stone, *Carbohydr. Res.*, 127 (1984) 59–73.
16 A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
17 L. Braunsweiler and R.R. Ernst, *J. Magn. Reson.*, 53 (1983) 521–526.
18 D.G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 2820–2821.
19 M.W. Edwards and A. Bax, *J. Am. Chem. Soc.*, 108 (1986) 918–923.
20 A. Bax and D.G. Davis, *J. Magn. Reson.*, 63 (1985) 207–213.
21 D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
22 K. Bock and H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
23 P.A.J. Gorin and J.F.T. Spencer, *Can. J. Chem.*, 43 (1965) 2978–2984.
24 J. Kärkkäinen, *Carbohydr. Res.*, 14 (1970) 27–33.
25 J. Kärkkäinen, *Carbohydr. Res.*, 17 (1971) 11–18.
26 R.U. Lemieux, K. Bock, L.T.J. Delbaere, S. Koto, and V.S. Rao, *Can. J. Chem.*, 58 (1980) 631–653.
27 V.K. Dua, B.N.N. Rao, S-S. Wu, V.E. Dube, and C.A. Bush, *J. Biol. Chem.*, 261 (1986) 1599–1608.
28 C.A. Bush, Z-Y. Yan, and B.N.N. Rao, *J. Am. Chem. Soc.*, 108 (1986) 6168–6173.