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STRUCTURAL STUDIES ON A CELL WALL POLYSACCHARIDE PREPARATION OF *LACTOCOCCUS LACTIS* SUBSPECIES *CREMORIS* H414

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ABSTRACT

A cell wall polysaccharide preparation of *Lactococcus lactis* subsp. *cremoris* H414 was isolated by nitrous acid extraction, and purified by gel filtration and anion-exchange chromatography. Oligosaccharide fragments, obtained by partial acid hydrolysis or alkaline hydrolysis, were studied by means of monosaccharide analysis, methylation analysis, FAB-MS, EI-MS, and ^{1}H NMR spectroscopy. In addition ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, including two-dimensional homo- and heteronuclear measurements, were performed on the native polysaccharide. Combination of the various results demonstrated that in the polysaccharide preparation two repeating units with the structures [→2)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→]$_n$ and [→6)-[α-D-Gal*p*-(1→4)]-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-Ribitol-(5-phosphate→]$_m$, respectively, can be recognized. So far, a covalent linkage between both polymers could not be confirmed nor be excluded.

INTRODUCTION

Lactococci are important starter cultures for fermentation in the dairy industry. One of the problems encountered during the manufacturing of the fermented milk products is

bacteriophage infection which can result in lysis of the starter culture. The first stage in bacteriophage infection is a specific recognition between the phage and the host cell. In Gram-negative bacteria the phage receptors are lipopolysaccharides or specific proteins in the outer membrane, whereas in Gram-positive bacteria cell wall carbohydrates are almost always involved in phage adsorption.[1]

Only little attention has been paid to the biochemical characterisation of the phage receptors of the Gram-positive lactic acid bacteria. For *Lactococcus* species, phage receptors have been found on cell wall material, and they are probably part of the peptidoglycan or the group-specific carbohydrate.[2-4] So far, an exception is the receptor for phage ml3, which has been shown to be a membrane protein.[5] The phage binding ability of *Lactococcus lactis* subsp. *cremoris* SK112 was reduced significantly by Concanavalin A.[3] In the case of *L. lactis* subsp. *cremoris* EB7 it was observed that a mixture of D-galactosamine and L-rhamnose reduced the binding of phages.[4] Furthermore, it has been found that the bacteriophage kh receptor of *L. lactis* subsp. *cremoris* KH is a rhamnose constituent of the cell wall polysaccharide.[1] Phage adsorption to *L. lactis* subsp. *cremoris* Wg2-1 takes place only on a limited number of receptor sites on the cell wall, whereas for *L. lactis* subsp. *diacetyllactis* F7/2 a uniform adsorption to the whole cell surface was observed.[6] In both cases treatment of cell walls with lysozyme, metaperiodate or acid resulted in inhibition of phage binding, suggesting that a carbohydrate component is part of the phage receptors.

In this report structural studies on a cell wall polysaccharide preparation of *L. lactis* subsp. *cremoris* H414 are described.

RESULTS AND DISCUSSION

Composition of the cell wall polysaccharide preparation. After gel filtration (Sephacryl S-100) and anion-exchange chromatography (Q Sepharose) of the crude extract, about 90 mg cell wall polysaccharide, free of protein, was obtained from 3 g of lyophilised cells. The molecular mass of the polysaccharide, as determined on Superose 12 HR, is approximately 14 kDa. Monosaccharide analysis with *N*-acetylation, including absolute configuration determination, of the native polysaccharide showed the presence of L-rhamnose, D-galactose, *N*-acetyl-D-galactosamine, and 2,5-anhydro-ribitol in a molar ratio of 1.1:1.0:0.9:0.4 (Table 1). The finding of 2,5-anhydro-ribitol suggested the presence of ribitol-phosphate as a constituent of the polysaccharide (compare with the analysis of teichoic acids[7,8]). Monosaccharide analysis after solvolysis with anhydrous HF yielded a relative increase in the *N*-acetylgalactosamine content (0.9→1.4; Table 1).

TABLE 1. Monosaccharide Composition of the Polysaccharide (PS) and of Oligosaccharide Fractions obtained by Alkaline Hydrolysis (A, B).

Monosaccharide	Compound									
	PS[a]	PS[b]	A	A1	A2	B	B1	B1[c]	B2	B2[c]
Rha	1.1	1.2	1.1	1.2	1.1	+				
Gal	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
GalNAc	0.9	1.4						1.9		1.8
Rib-ol[d]	0.4	0.5	+		0.1	0.6	1.0	0.5	0.6	0.6

a. Monosaccharide composition after methanolysis. b. Monosaccharide composition after solvolysis with anhydrous HF, followed by methanolysis. c. Before methanolysis, a *N*-acetylation step with acetic anhydride was performed. d. In all monosaccharide analyses ribitol was present as 2,5-anhydro-ribitol.

In the 500 MHz ^1H NMR spectrum of the polysaccharide (Fig. 1) five H-1 signals of nearly equal intensity were observed at δ 5.198 (s), 5.116 ($J_{1,2}$ = 3.8 Hz), 5.081 ($J_{1,2}$ = 3.5 Hz), 4.998 ($J_{1,2}$ = 4.1 Hz), and 4.589 ($J_{1,2}$ = 8.5 Hz), respectively. The CH_3-signal of rhamnose was detected at δ 1.314 ($J_{5,6}$ = 6.1 Hz), whereas two signals of equal intensity for *N*-acetyl methyl protons were present at δ 2.096 and 2.037, respectively. The ^{13}C NMR spectrum (Fig. 2) showed five anomeric C-signals of equal intensity at δ 102.46, 101.82, 100.68, 98.75, and 94.75, respectively, a CH_3-signal of rhamnose at δ 17.87, two *N*-acetyl CO-signals at δ 176.14 and 176.01, respectively, and two *N*-acetyl CH_3-signals at δ 23.76 and 23.32, respectively. On the basis of detailed NMR studies on the native polysaccharide (see below) it can be deduced that all monosaccharide residues occur in the pyranosidic ring form. The NMR data suggest the presence of rhamnose, galactose and *N*-acetylgalactosamine in a molar ratio of 1:2:2, which contrast the GLC data mentioned above. The low molar ratio of *N*-acetylgalactosamine and anhydro-ribitol as found in the monosaccharide analysis can be explained by incomplete hydrolysis of the glycosidic linkages of the *N*-acetylgalactosaminyl residues (see below). However, for the low molar ratio of galactose in the monosaccharide analysis, as compared to the NMR analyses, no explanation can be given so far.

The phosphorus content of 2 % corresponds with 0.7 mol phosphate/mol repeating unit, taking into account a repeating unit composed of one rhamnosyl, one ribitol-phosphate, two galactosyl, and two *N*-acetylgalactosaminyl residues.

Methylation analysis. Methylation analysis of the native polysaccharide revealed as major neutral residues a 2-linked rhamnopyranosyl and a 3-linked galactopyranosyl residue (Table 2). Furthermore, substantial amounts of a terminal galactopyranosyl and a 3-

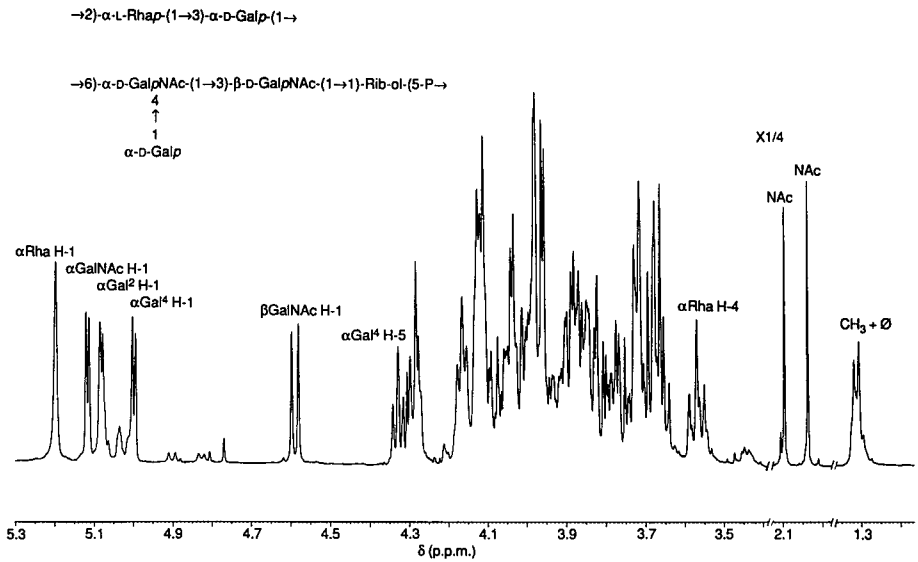

FIG. 1. 500 MHz ^{1}H NMR spectrum of the native polysaccharide recorded at 27 °C. The resonance marked with a ø stems from a non-protein, non-carbohydrate contaminant.

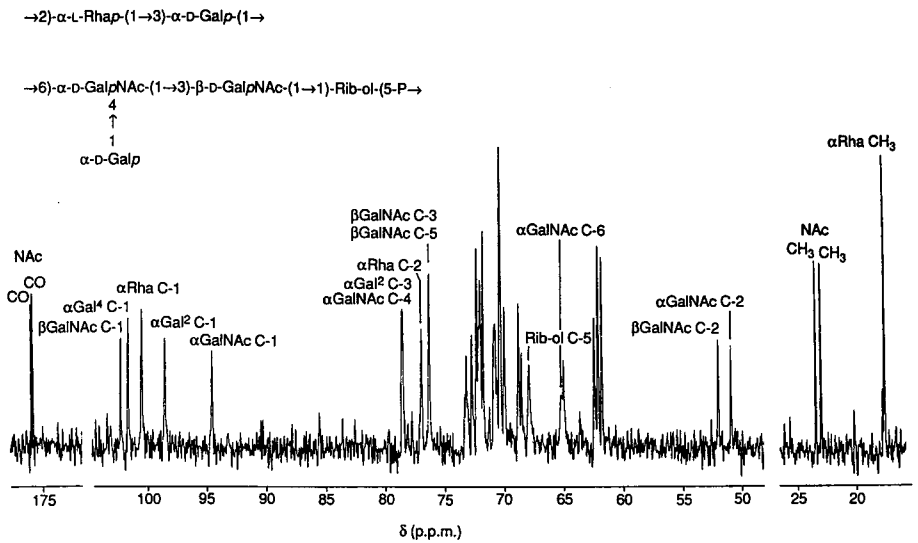

FIG. 2. 50 MHz ^{13}C NMR spectrum of the native polysaccharide recorded at 27 °C.

TABLE 2. Methylation Analysis Data of the Native Polysaccharide and Fraction A1 obtained from the Alkaline Hydrolysate after Gel Filtration and Anion-exchange Chromatography.

Compound	Derivative				
	3,4-Rha[a]	2,4-Rha[a]	2,3,4,6-Gal[a]	2,4,6-Gal[a]	2,3,4-Rib[b]
Native PS	0.9	0.3	0.4	1.0	+
A1	1.3	0.1		1.0	

a. 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol-1-*d*, etc. b. 2,3,4-Rib = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-ribitol.

substituted rhamnopyranosyl residue were found. The occurrence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-ribitol suggested a 1,5-disubstituted ribitol unit.

Partial acid hydrolysis. To generate oligosaccharide fragments for structural analysis, the polysaccharide was treated with 48% aqueous HF, and the obtained hydrolysate was fractionated on Bio-Gel P-6 yielding fractions I-VI (Fig. 3).

The major fraction III was subfractionated on Bio-Gel P-2, affording one major component, which was shown to be homogeneous by HPLC on Lichrosorb-NH_2. Monosaccharide analysis of this component, as established by GLC of the trimethylsilylated methyl glycosides, revealed the presence of galactose and *N*-acetylgalactosamine in a molar ratio of 1.0:1.7. After reduction with $NaBD_4$, yielding compound **1**, galactose, *N*-acetylgalactosamine, and *N*-acetylgalactosaminitol were found in a molar ratio of 1.0:0.8:0.5. In the 360 MHz ^1H NMR spectrum of **1** two α anomeric signals were observed at δ 5.153 ($J_{1,2}$ = 3.6 Hz) and 5.031 ($J_{1,2}$ = 3.7 Hz), respectively. The positive-ion FAB-MS spectrum of permethylated **1** showed a pseudomolecular ion $[M+H]^+$ at *m/z* 758, in agreement with the occurrence of one hexosyl, one *N*-acetylhexosaminyl, and one *N*-acetylhexosaminitol-*1-d* residue, and fragment ions at *m/z* 219 (→*m/z* 187), *m/z* 464 (→*m/z* 432), *m/z* 277, and *m/z* 522, indicating the sequence hex-hexNAc-hexNAc-ol-*1-d* . GLC-(EI)MS of permethylated **1** yielded the same series of fragment ions (*m/z* 219, aA_1; *m/z* 187, aA_2; *m/z* 464, baA_1; *m/z* 432, baA_2; *m/z* 277, cA_1; *m/z* 522, bcA_1; *m/z* 490, bcA_2). The fragment ions *m/z* 133 ($CH_2OMeCHOMeCHOMe$) and *m/z* 624 (M – $CH_2OMeCHOMeCHOMe$), formed by cleavage of the C-3–C-4 bond of the alditol, are indicative of a 1→3 linkage between the hexNAc and hexNAc-ol residue. The presence of the fragment ion at *m/z* 129 (CHOMeCHNAcMe; bH_1[2]) together with the characteristic, relatively intense, bE_3 fragment ion *m/z* 182 suggest a 1→4 or a 1→6 linkage between hex and hexNAc[9] (for a 1→3

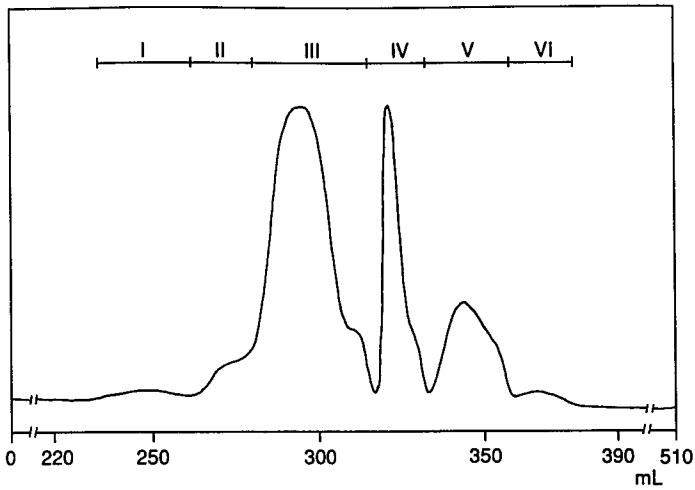

FIG. 3. Bio-Gel P-6 elution profile of the saccharide mixture obtained after partial acid hydrolysis of the native polysaccharide with 48% aqueous HF.

linkage the latter fragment should have been observed at *m/z* 386). No discrimination between a 1→4 or a 1→6 linkage can be made on basis of the mass spectrum, and therefore the following structure for **1** is proposed:

α-D-Gal*p*-(1→4/6)-α-D-Gal*p*NAc-(1→3)-GalNAc-ol-*1-d*

1

Subfractionation of Bio-Gel P-6 fraction II on Bio-Gel P-2 yielded one major component **2**, which behaved as a neutral substance on Mono Q. The 500 MHz ^{1}H NMR spectrum of **2** (Fig. 4) showed three anomeric signals of equal intensity at δ 5.114 ($J_{1,2}$ = 3.8 Hz), 5.005 ($J_{1,2}$ = 4.0 Hz), and 4.575 ($J_{1,2}$ = 8.5 Hz), respectively, and two *N*-acetyl methyl signals at δ 2.063 and 2.039, respectively. Monosaccharide analysis of **2** revealed the presence of galactose, *N*-acetylgalactosamine, and ribitol in a molar ratio of 1.0:0.9:0.5. Combination of the NMR and GLC data, taking into account incomplete hydrolysis of glycosidic linkages of *N*-acetylgalactosaminyl residues and the structure of **1**, supports a tetrasaccharide-alditol structure for **2**, with one α-galactosyl, one α-*N*-acetylgalactosaminyl, one β-*N*-acetylgalactosaminyl and one ribitol residue.

To determine the sequence of the residues and the linkage positions in **2**, 2D COSY, HOHAHA, and ROESY experiments were performed (data not shown). The H-1 signal at

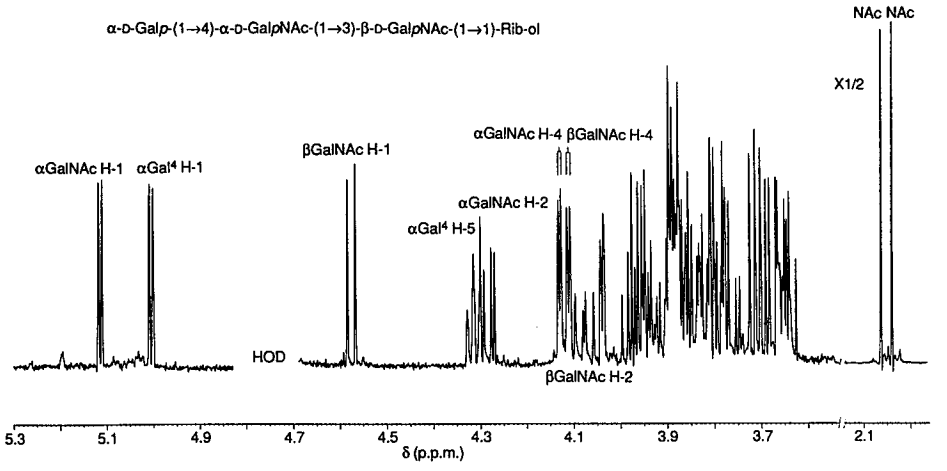

FIG. 4. 500 MHz ^{1}H NMR spectrum of the tetrasaccharide-alditol **2** obtained after gel filtration and HPLC on Lichrosorb-NH_2 of the Bio-Gel P-6 fraction II.

δ 5.114 was assigned to α-*N*-acetylgalactosamine on account of the chemical shift of its H-2 at δ 4.284. This implies that the H-1 signal at δ 5.005 can be assigned to α-galactose, whereas the H-1 signal at δ 4.575 is attributed to the β-*N*-acetylgalactosaminyl residue. The non-anomeric proton assignments of these residues and the proton assignments of the ribitol residue, as presented in Table 3, are based on cross-peaks observed in the various 2D spectra. The sequence αGal→αGalNAc→βGalNAc→Rib-ol followed from the inter-residual nOe's αGal H-1,αGalNAc H-4; αGalNAc H-1,βGalNAc H-4 (strong), H-3 (weak); and βGalNAc H-1,Rib-ol H-1a or 1b observed in the ROESY spectrum. For blood group A-containing compounds it has been observed that the α-GalNAc-(1→3)-Gal linkage gives rise to a strong nOe between H-1 of α-GalNAc and H-4 of Gal.[10-12] Taking into account this observation together with the GLC-(EI)MS data of permethylated **1**, the linkages for αGal→αGalNAc and αGalNAc→βGalNAc were established as 1→4 and 1→3, respectively. The inter-residual nOe βGalNAc H-1,Rib-ol H-1a or 1b indicated a 1→1 linkage, which is in agreement with the methylation analysis data of the native polysaccharide (Table 2), showing a 1,5-disubstituted ribitol. Therefore, the structure of **2** is as follows:

α-D-Gal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol

2

TABLE 3. ^{1}H NMR Chemical Shifts[a] of the Tetrasaccharide-alditol 2 and the Native Polysaccharide PS.

Compound	Proton	Residue[b]					
		αGal^4	αGalNAc	βGalNAc	Rib-ol	αRha	αGal^2
2[c]	H-1(a)	5.005	5.114	4.575	3.98[d]		
	H-1b				3.869[d]		
	H-2	3.860	4.284	4.076	3.94		
	H-3	3.965	3.889	3.822	3.715		
	H-4	4.039	4.129	4.110	3.79		
	H-5	4.314		3.653	3.645[e]		
	H-6a[f]	3.709		3.835			
	H-6b[f]	3.665		3.765			
	NAc[g]		2.039	2.063			
PS[h]	H-1(a)	4.998	5.116	4.589	3.984[d]	5.198	5.081
	H-1b				3.886[d]		
	H-2	3.878	4.294	4.097	3.93[i]	4.131	3.955
	H-3	3.97	3.889	3.835	3.78	3.969	4.036
	H-4	4.037	4.128	4.105	3.985[i]	3.566	4.115
	H-5(a)	4.328	4.015	3.67	4.067[j]	3.857	4.28
	H-5b				3.995[j]		
	H-6a[f]	3.702	4.165	3.83			3.724
	H-6b[f]	3.661	4.165	3.76			3.724
	CH_3					1.314	
	NAc[g]		2.037	2.096			

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. b. The superscript indicates to which position of the adjacent monosaccharide the Gal residue is glycosidically linked. c. Chemical shifts at 27 °C. d. Chemical shifts for H-1a and H-1b can be interchanged. e. Chemical shift for H-5a and/or H-5b. f. Chemical shifts for H-6a and H-6b can be interchanged. g. Assignments based on NMR data of the trisaccharide αGal1→4αGalNAc1→3α/βGalNAc. h. Chemical shifts at 32 °C, except for the anomeric protons, αGal^4 H-5, αGalNAc H-6a/6b, Rib-ol H-5a/5b, αRha H-4, and CH_3, and the *N*-acetyl methyl protons, of which the chemical shifts are given at 27 °C. i,j. Chemical shifts can be interchanged.

Alkaline hydrolysis. Treatment of the polysaccharide with alkali, followed by gel filtration on Bio-Gel P-2 afforded two major carbohydrate-containing fractions, A and B, respectively. Monosaccharide analysis of A revealed the presence of rhamnose and galactose in a molar ratio of 1.1:1.0 and a trace of 2,5-anhydro-ribitol (Table 1). Further purification by anion-exchange chromatography on Mono Q, yielded a major component A1, eluting in the neutral region, and a minor component A2, eluting in the acidic region. Monosaccharide analysis showed for both fractions the presence of rhamnose and galactose in nearly equal amounts, and for A2 also a relatively small amount of 2,5-anhydro-ribitol

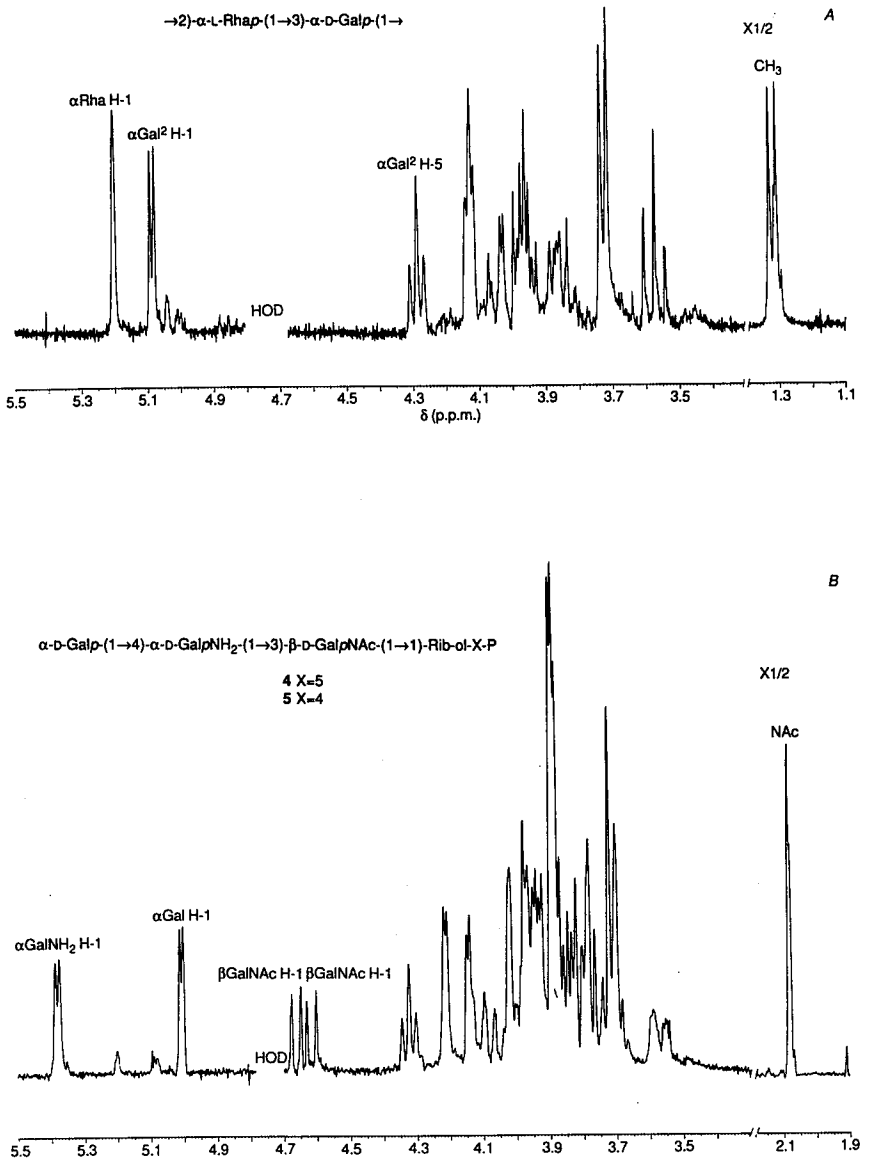

FIG. 5. (*A*). 300 MHz ^{1}H NMR spectrum of fraction A1 obtained from the alkaline hydrolysate after gel filtration on Bio-Gel P-2 and anion-exchange chromatography on Mono Q; (*B*). 300 MHz ^{1}H NMR spectrum of fraction B1 obtained from the alkaline hydrolysate after gel filtration on Bio-Gel P-2 and anion-exchange chromatography on Mono Q.

(Table 1). The 300 MHz ^{1}H NMR spectrum of A1 (Fig. 5A) showed two H-1 signals at δ 5.202 (s) and 5.085 ($J_{1,2}$ = 4.0 Hz), belonging to the rhamnosyl and the galactosyl residue, respectively. The CH_3-signal of rhamnose was observed at δ 1.319, whereas the H-5 signal of galactose occurred at δ 4.287. In the methylation analysis two major residues, a 2-linked rhamnosyl and a 3-linked galactosyl residue, respectively, and a minor 3-linked rhamnosyl residue were found (Table 2). The occurrence of a 3-linked rhamnosyl residue was also observed in the methylation analysis of the native polysaccharide (Table 2), but could not be explained. On basis of these results a tentative structure for a disaccharide repeating unit **3** is proposed:

→2)-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→

3

Monosaccharide analysis of fraction B indicated the presence of galactose and 2,5-anhydro-ribitol in a molar ratio of 1.0:0.6, and a trace of rhamnose (Table 1). Using anion-exchange chromatography on Mono Q, fraction B was separated into a major fraction B1, eluting in the neutral region, and a minor fraction B2, eluting in the acidic region, both containing galactose and 2,5-anhydro-ribitol (Table 1). Moreover, when an *N*-acetylation step with acetic anhydride was applied before the monosaccharide analysis, also *N*-acetylgalactosamine was observed (Table 1), indicating the presence of galactosamine in both B1 and B2. In the 300 MHz ^{1}H NMR spectrum of B1 (Fig. 5B) four H-1 signals at δ 5.381 ($J_{1,2}$ = 3.5 Hz), 5.007 ($J_{1,2}$ = 3.0 Hz), 4.661 ($J_{1,2}$ = 8.4 Hz), and 4.615 ($J_{1,2}$ = 8.5 Hz), respectively, and an *N*-acetyl methyl signal at δ 2.081 were observed in a molar ratio of 2:2:1:1:6, suggesting a mixture of two closely related components. By comparison with the ^{1}H NMR spectrum of oligosaccharide **2** the signal at δ 5.007 was identified as αGal^4 H-1. It has been reported that during treatment with alkali *N*-deacetylation of *N*-acetylhexosamines takes place, except for 3-substituted *N*-acetylhexosaminyl residues.[13] Therefore, the two β H-1 signals (and the *N*-acetyl methyl signal) were assigned to two β-*N*-acetylgalactosaminyl residues each belonging to a different compound, and the signal at δ 5.381 to H-1 of α-galactosamine.

HPAEC-PAD of B1 resulted in two fractions B1.1 and B1.2, respectively. In the negative-ion FAB mass spectrum of B1.1 a pseudomolecular ion $[M-H]^-$ was observed at *m/z* 757, in agreement with a tetrasaccharide-alditol **4** (or **5**) containing one galactosyl, one galactosaminyl, one *N*-acetylgalactosaminyl and one ribitol-phosphate residue. In the negative-ion FAB mass spectrum of B1.2 the same pseudomolecular ion was observed, indicating the presence of a closely related tetrasaccharide-alditol **5** (or **4**). Taking into account

the ^{1}H NMR spectrum of the mixture, it is tempting to assume that the only difference in structure between **4** and **5** is located in the ribitol-phosphate unit, resulting in a different chemical shift for H-1 of the two β-*N*-acetylgalactosaminyl residues. It has to be noted that the cleavage of phosphodiester linkages proceeds via a cyclic phosphate intermediate[14] involving two neighbouring carbon atoms. Subsequently, the intermediate is hydrolysed to a phosphomonoester. The cyclic intermediate can be opened in two different ways, resulting in different phosphomonoesters. Apparently, this occurred during the alkaline hydrolysis of the polysaccharide, giving rise to two tetrasaccharide-alditols with phosphate located on two different positions in the ribitol unit. Taking into account the structure of tetrasaccharide-alditol **2** and the presence of a 1,5-disubstituted ribitol in the native polysaccharide (Table 2), the following structures are proposed for the two tetrasaccharide-alditols **4** and **5**:

α-D-Gal*p*-(1→4)-α-D-Gal*p*NH_2-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol-X-phosphate

4 X=5; **5** X=4

NMR spectroscopy of the native polysaccharide. Based on the foregoing, for the native acidic polysaccharide preparation a combination of two repeating structural elements could be established, namely, a tetrasaccharide-alditol repeating unit **2** with phosphate linked to ribitol (**4**) and a disaccharide repeating unit **3**. However, no overlapping elements were found in the partial acid and alkaline hydrolysates. Therefore, 2D NMR techniques were applied on the native polysaccharide in a search for (i) the linkage between both units, (ii) the anomeric configuration of the rhamnosyl unit, and (iii) the monosaccharide residue to which the phosphate group is linked.

The assignment of the H-1 signals in the ^{1}H NMR spectrum of the polysaccharide (Fig. 1) was performed on guidance of the ^{1}H NMR data of **2** (Table 3, Fig. 4) and **3** (Fig. 5A). The assignments of all the other proton resonances, except H-5a,5b of the ribitol unit, were achieved via cross-peaks observed in the 2D COSY, HOHAHA, and ROESY spectra (data not shown), and the chemical shifts are compiled in Table 3. The α configuration of the rhamnosyl residue was deduced from the position of the H-5 signal at δ 3.857 (compare with H-5 of α-L-Rha-OMe and β-L-Rha-OMe at δ 3.67 and 3.39, respectively[15]), from the observation of only one intra-residual nOe, namely, Rha H-1,Rha H-2, and from the $J_{C1,H1}$ value of 170 Hz obtained from the HMQC-NOE experiment (see below).[16-18]

In the ^{31}P NMR spectrum one multiplet at δ 1.284 was observed, indicating that only one type of phosphate is present in the polysaccharide. The SED spectrum, containing

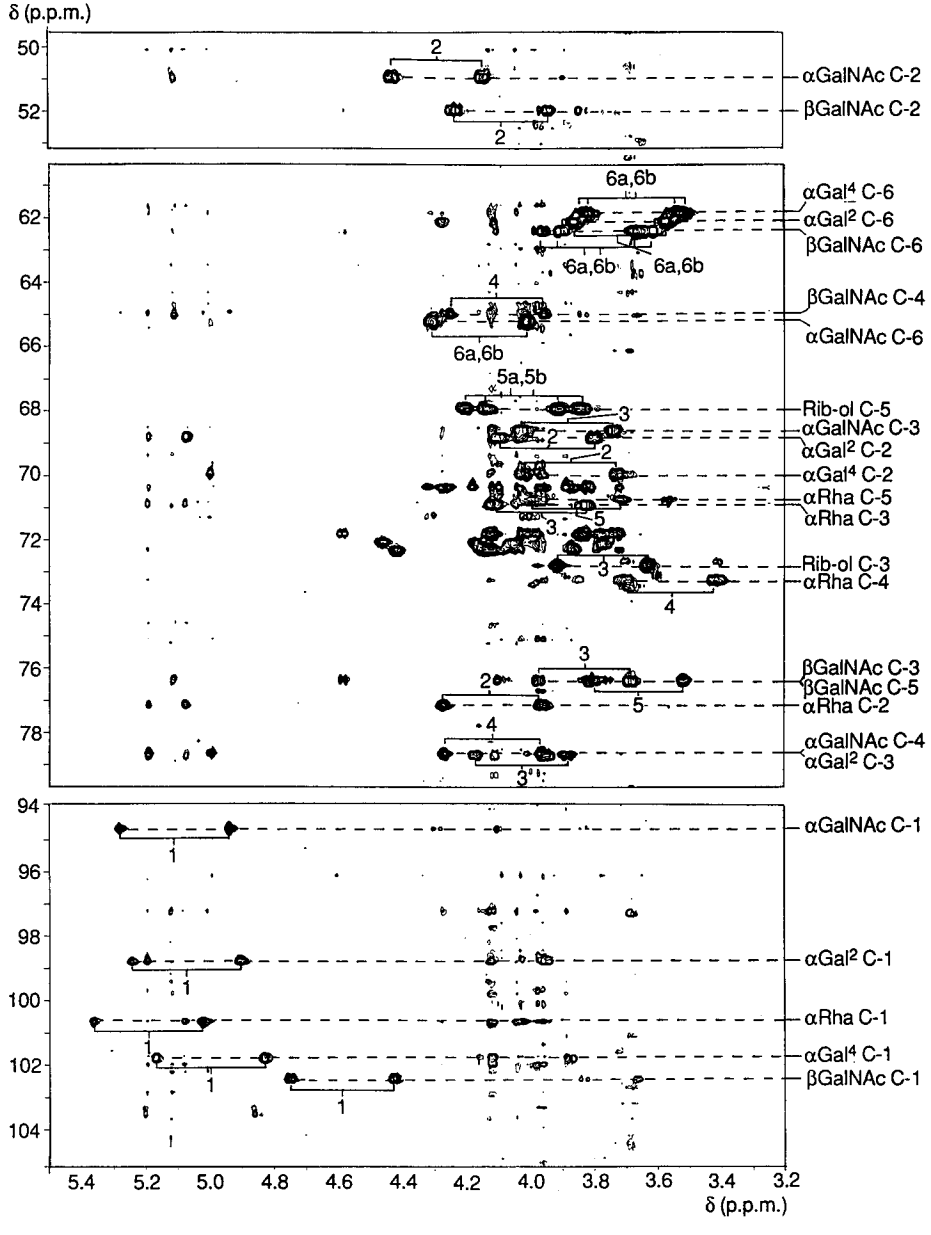

FIG. 6. HMQC-NOE $^{1}H/^{13}C$ spectrum of the native polysaccharide. Only cross-peaks of carbon atoms with their corresponding protons are indicated.

TABLE 4. ^{13}C NMR Chemical Shifts[a] of the Native Polysaccharide.

Carbon atom	Residue					
	αGal^4	αGalNAc	βGalNAc	Rib-ol	αRha	αGal^2
C-1	101.82 (J 170)[b]	94.75 (J 172)	102.46 (J 160)	71.76 (Δδ +8.1)[c]	100.68 (J 170) (Δδ -1.5)	98.75 (J 170)
C-2	69.96	50.93 (Δδ -3.9)	51.99 (Δδ -1.4)	72.00[d] (Δδ +5.5)	77.00 (Δδ -0.7)	68.78
C-3	70.32	68.54 (Δδ -1.1)	76.34 (Δδ +3.0)	72.71 (Δδ -0.5)	70.75 (Δδ +7.8)	78.60
C-4	70.32	78.60 (Δδ +8.2)	65.00 (Δδ -5.0)	71.76[d] (Δδ -1.6)	73.17 (Δδ -0.2)	70.32
C-5	72.00	72.30 (Δδ -0.3)	76.34	67.88 (Δδ +4.2)	70.75	72.30
C-6	61.78	65.28 (Δδ +2.2)	62.42		17.87	62.12

a. In ppm relative to internal acetone at δ 31.55 in D_2O at 27 °C. b. $J_{C1,H1}$ in Hz. c. Chemical shift differences as compared to the corresponding methyl glycosides, the monosaccharide βGalNAc or Rib-ol. d. Chemical shifts can be interchanged.

only POC*H* signals, showed three ^1H signals at δ 4.165, 4.067, and 3.995 in an intensity ratio of 2:1:1. The signal at δ 4.165 corresponds with H-6a and H-6b of αGalNAc (Table 3), establishing a phosphate-(1→6)-αGalNAc linkage. The two signals at δ 4.067 and 3.995 were identified as H-5a and H-5b of Rib-ol, based on the fact that the phosphate group is linked to C-5 of Rib-ol, as determined by methylation analysis of the polysaccharide (Table 2).

Analysis of the inter-residual nOe's in the ROESY spectrum supported the presence of a tetrasaccharide-alditol phosphate repeating unit (αGal^4 H-1,αGalNAc H-4; αGalNAc H-1,βGalNAc H-4 and H-3; βGalNAc H-1,Rib-ol H-1a and H-1b) and a disaccharide repeating unit (αRha H-1,αGal^2 H-1,H-3,H-2 and αGal^2 H-1,αRha H-1,H-2). However, no connectivities could be observed between residues of the two repeating elements.

A two-dimensional HMQC-NOE $^1H/^{13}C$ experiment was carried out to obtain a complete assignment of the ^{13}C NMR spectrum (Fig. 2). In the HMQC-NOE spectrum (Fig. 6) the cross-peaks of the protons with their corresponding carbon atoms (HMQC cross-peaks) are doubled due to the ^1H-^{13}C coupling, whereas the nOe cross-peaks are single peaks. The ^{13}C signals were assigned on guidance of HMQC cross-peaks and relevant nOe cross-peaks, and the chemical shifts are listed in Table 4. The nOe's αRha C-1, αGal^2 H-1,H-2,H-3; αGal^2 C-1,αRha H-1,H-2; αGal^4 C-1,αGalNAc H-4; αGalNAc

C-1,βGalNAc H-3,H-4; and Rib-ol C-1,βGalNAc H-1 confirmed the sequence of the residues in the two repeating units. For substituted carbon atoms downfield shift effects of ≈ 5-10 ppm have been observed, and for the adjacent carbon atoms upfield shift effects of ≈ 0-3 ppm.[19] Comparison of the ^{13}C chemical shifts for the native polysaccharide with those for α-Rha-OMe,[20] α-GalNAc-OMe,[21] α-GalOMe,[20] β-GalNAc,[21] and Rib-ol[20] showed downfield effects for αRha C-2, αGal[2] C-3, αGalNAc C-4, βGalNAc C-3, and Rib-ol C-1, and upfield shift effects for their adjacent carbon atoms (Table 4). The downfield shift effects for Rib-ol C-5 and αGalNAc C-6 (Table 4) and the broadening of their signals, due to ^{31}P-^{13}C coupling, are in agreement with the Rib-ol-(5-phosphate→6)-αGalNAc linkage.

In conclusion, the structures of the two repeating units are as follows:

→2)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→

6

→6)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol-(5-phosphate→
4
↑
1
α-D-Gal*p*

7

Both in the ROESY spectrum and in the HMQC-NOE spectrum of the native polysaccharide preparation no indications were found for a linkage between the disaccharide repeating unit **6** and the tetrasaccharide-alditol repeating unit **7**. The possibility of a mixture of two polysaccharides is unlikely because anion-exchange chromatography should have resulted in the separation of the polysaccharide containing the neutral disaccharide repeating unit and the polysaccharide containing the negatively charged tetrasaccharide alditol repeating unit. Further investigations will be necessary to unravel the association/linkage between the established polysaccharide structures.

EXPERIMENTAL

Bacterial growth and isolation of cell wall polysaccharide. *Lactococcus lactis* subsp. *cremoris* H414 was grown in defined medium[22,23] with lactose as carbon source for 48 h at pH 7.0 and 30 °C. The cells were collected by centrifugation at 13,000

g, washed two times with water, followed by centrifugation, and lyophilisation. Cell wall polysaccharide material was isolated according to Ref. 24. Lyophilised cells (3 g) were suspended in water (90 mL), and 4M $NaNO_2$ (30 mL), glacial acetic acid (30 mL), and a few drops of 1-octanol were added. After stirring the mixture for 1 h at room temperature, the cells were removed by centrifugation, and the supernatant was dialysed against running tap water (24 h) and bidistilled water (24 h, 5 x 5 L), followed by lyophilisation. The material was purified by gel filtration on a column (120 x 2.2 cm) of Sephacryl S-100 (Pharmacia), using 50 mM NH_4HCO_3 as eluent (30 mL/h, 5-mL fractions), and UV monitoring at 206 nm (LKB 2238 Uvicord SII). The polysaccharide-containing fraction (orcinol-sulfuric acid test) was further purified by anion-exchange chromatography on a column (10 x 1.6 cm) of Q Sepharose Fast Flow (Pharmacia). After washing with water (30 mL), a linear gradient from 0–0.1M NaCl in 240 mL water was applied, followed by a steeper gradient from 0.1–1.0M NaCl in 240 mL water, and finally M NaCl (30 mL) was used. The flow rate was 4 mL/min and the eluate was monitored by UV detection at 214 nm (Pharmacia UV-1/214). After lyophilisation of the polysaccharide-containing fraction (orcinol-sulfuric acid test; 0.25M NaCl), NaCl was removed by gel filtration on a column (45 x 1.5 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad), using water as eluent and refractive index monitoring (Bischoff RI-detector 8100).

Monosaccharide analysis. Samples (0.1–0.5 mg) were subjected to methanolysis (methanolic M HCl, 24 h, 85 °C), and the trimethylsilylated *N*-(re)acetylated methyl glycosides were analysed on an SE-30 fused-silica capillary column (25 m x 0.32 mm, Pierce), using a Varian 3700 gas chromatograph (temperature program 130→220 °C at 4 °C/min).[25] The absolute configuration of the monosaccharides was determined by GLC of the trimethylsilylated *N*-(re)acetylated (–)-2-butyl glycosides.[26,27]

Solvolysis with anhydrous hydrogen fluoride. Dried polysaccharide (8 mg) was dissolved in 4 mL anhydrous HF and kept for 3 h at room temperature. After evaporation of HF, the residue was treated with 2M trifluoroacetic acid for 3 h at 120 °C, and then the acid was removed by co-evaporation with water. One-quarter of the residue was dissolved in 1 mL saturated $NaHCO_3$, and five aliquots of 10 µL acetic anhydride were added at 5-min intervals. After 1 h at room temperature, the mixture was desalted on a column (5 x 0.7 cm) of Dowex AG 50W-X8 (H^+) resin (100–200 mesh, Bio-Rad), and lyophilised. The residue was subjected to monosaccharide analysis.

Protein assay. Protein content was determined with the Pierce Protein Assay Reagent, and bovine serum albumin was used as a standard.

Phosphate determination. Phosphate was determined according to Ref. 28.

Molecular mass determination. The molecular mass of the polysaccharide was determined by gel filtration on a column of Superose 12 HR 10/30 (Pharmacia FPLC sys-

tem), using 100mM NH_4HCO_3 as eluent (0.5 mL/min), and refractive index monitoring. The column was calibrated with pullulan standards (Machery-Nagel) from 5.8 kDa up to 853 kDa.

Partial acid hydrolysis. Polysaccharide (10 mg) was treated with 48% aqueous HF (2 mL) for 4 days at –16 °C. Then, the solvent was evaporated in a vacuum desiccator over solid sodium hydroxide, and the residue was fractionated on a column (134 x 2.2 cm) of Bio-Gel P-6 (100–200 mesh, Bio-Rad), using 50mM NH_4HCO_3 as eluent (20 mL/h) and UV monitoring at 206 nm. Carbohydrate-containing fractions (orcinol-sulfuric acid test) were separated on a column (90 x 1.5 cm) of Bio-Gel P-2, using 50mM NH_4HCO_3 as eluent (15 mL/h) and UV monitoring/orcinol-sulfuric acid colorimetry.

Alkaline hydrolysis. Polysaccharide (7 mg) was dissolved in 1.5 mL 2M NaOH, and after the addition of 1 mg $NaBH_4$, the solution was kept for 4 h at 100 °C. The hydrolysate was neutralised with 2M HCl and fractionated on a column (95 x 1.2 cm) of Bio-Gel P-2, using 5mM NH_4HCO_3 as eluent (9 mL/h, 1.1-mL fractions), and refractive index monitoring/orcinol-sulfuric acid colorimetry.

Methylation analysis. Samples were permethylated as reported in Ref. 29. After hydrolysis with 90% formic acid (1 h, 100 °C) and 4M trifluoroacetic acid (4 h, 100 °C), the partially methylated monosaccharides were reduced with $NaBD_4$, followed by acetylation.[30] The partially methylated alditol acetates were analysed by GLC on a CPsil 43 WCOT fused-silica capillary column (25 m x 0.32 mm, Chrompack), using a Varian 3700 gas chromatograph (temperature program 170→220 °C at 4 °C/min, followed by 15 min at 220 °C), and by GLC-MS using a Carlo Erba GC/Kratos MS80/Kratos DS 55 instrument (electron energy, 70 eV; accelerating voltage, 2.7 kV; ionising current, 100 mA; CPsil 43 capillary column).

High performance liquid chromatography. HPLC was carried out on a Kratos liquid chromatograph, consisting of two Spectroflow 400 solvent delivery systems, a Spectroflow 450 solvent programmer, and a Rheodyne injection valve module using a Lichrosorb-10 NH_2 column (250 x 4.6 mm). Separations were performed isocratically with a mixture of 80:20 acetonitrile-water at a flow rate of 1.0 mL/min and UV monitoring at 206 nm (Spectroflow 783 programmable absorbance detector).

Medium pressure anion-exchange chromatography. Medium pressure anion-exchange chromatography using a Pharmacia FPLC system was performed on a Mono Q HR 5/5 column. Elutions were carried out with water (2 mL), followed by a linear gradient from 0–0.1M NaCl in 8 mL water, a steeper gradient from 0.1–1.0M NaCl in 8 mL water, and finally by M NaCl (2 mL). The carbohydrate-containing fractions (orcinol-sulfuric acid test) were desalted on a column (95 x 1.2 cm) of Bio-Gel P-2, using 2.5mM

NH_4HCO_3 as eluent (9 mL/h, 1.1-mL fractions), and refractive index monitoring. Residual NH_4HCO_3 was removed by repeated lyophilisation.

High pH anion-exchange chromatography - pulsed amperometric detection. HPAEC-PAD was carried out on a Dionex LC system, consisting of a Dionex BioLC quartenary gradient module, a model PAD-2 detector, and a CarboPac PA-1 pellicular anion-exchange column (250 x 9 mm), using the elution program 90:10 eluent A (0.1M NaOH)-eluent B (0.1M NaOH containing M NaOAc) for 0.3 min, then going to 1:1 eluent A-eluent B in 60 min at 5 mL/min and ambient temperature. Detection was made by PAD with a gold working-electrode and triple-pulse amperometry, comprising the following pulse potentials and durations: E_1 0.05 V and t_1 300 ms, E_2 0.65 V and t_2 60 ms, E_3 –0.95 V and t_3 180 ms; response time, 1 s. Data were collected and plotted by a Shimadzu C-R3A integrator. Fractions were neutralised immediately with M HCl, lyophilised, and desalted on a column (95 x 1.2 cm) of Bio-Gel P-2 using bidistilled water as eluent and refractive index monitoring.

Fast atom bombardment-mass spectrometry. Positive-ion FAB-mass spectra were recorded on a VG Analytical ZAB-HF mass spectrometer (Xe-beam, 7.6 keV; acceleration voltage, 8 kV). The carbohydrate samples were dissolved or dispersed in a glycerol matrix. Linear mass scans over 1500 daltons were recorded with an UV chart recorder. Negative-ion FAB-mass spectra were recorded on a Jeol AX 505W mass spectrometer (Xe-beam, 6 keV; acceleration voltage, 3 kV) equipped with a HP 9000 data system. The carbohydrate samples were dissolved or dispersed in a glycerol matrix (Bijvoet Center, Department of Mass Spectrometry, Utrecht University).

NMR spectroscopy. Samples were repeatedly exchanged in D_2O (99.9 atom% D, MSD Isotopes) with intermediate lyophilisation. Finally, the material was dissolved in 0.4 mL D_2O (99.96 atom% D).

The proton-decoupled ^{13}C NMR spectrum of the polysaccharide was recorded in D_2O at 27 °C on a Bruker WP 200 spectrometer equipped with a 10 mm broad-band probe. ^{13}C Chemical shifts are given in ppm downfield from the signal for external tetramethylsilane, but were actually measured by reference to internal acetone (δ 31.55). Resolution-enhanced ^1H NMR spectra were recorded at 27 °C on a Bruker AC-300, a Bruker HX-360 or a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, Utrecht University). ^1H Chemical shifts are given in ppm downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 27 °C).

2D Homonuclear Hartmann-Hahn (HOHAHA) spin-lock experiments were recorded at 27 °C (A) or 32 °C (B) using the pulse sequence 90°–t_1–SL–acq, wherein 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 was 65 (A) or 120 (B) ms. The spectral width was 1500 (A) or 2100 (B) Hz in each dimension.

2D Rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out at 27 °C (A) or 32 °C (B) using the pulse sequence $90_{\phi}{}^{o}$–t_1–SL–acq, wherein SL stands for a continuous spin-lock pulse of 200 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 was 2500 (A) or 4100 (B) Hz in each dimension.

For the HOHAHA and ROESY spectra 400 or 512 experiments of 2K data points were recorded. The time-proportional phase increment method[31] was used to create t_1 amplitude modulation. Each data matrix was zero-filled to 2K x 4K, and multiplied in each time domain with a phase-shifted sine function, shifted π/3 for the HOHAHA and π/2 for the ROESY, prior to phase-sensitive Ft.

The two-dimensional HMQC-NOE $^{1}H/^{13}C$ spectrum of the native polysaccharide was recorded on a Bruker AM-500 spectrometer with the pulse sequence as described in Ref. 32. Aquisition was preceded by four dummy scans, and 2048 t_1 increments of 16 scans each were recorded at a size of 2K, resulting in a total measuring time of approximately 20 h. The relaxation delay was 0.6 s; Δ, 1.78 ms; the 90° ^{1}H pulse width, supplied through the decoupling channel, 41 μs; the 90° ^{13}C pulse length, 11.3 μs; the spectral width in the ^{1}H time domain, 2100 Hz; the spectral width in the ^{13}C time domain, 11364 Hz; and the nOe mixing time, 250 ms. Cosine multiplications were used in the t_2 and t_1 domains. The resulting data set after Ft of 1024 x 1024 data points was baseline-corrected in both frequency domains by a third-order polynomal fit.

The ^{31}P NMR spectrum was recorded on a Bruker AM-500 spectrometer. ^{31}P Chemical shifts are expressed in ppm relative to the signal of orthophosphoric acid. The spin-echo difference (SED) spectrum was recorded with a 5 mm inverse broad-band probe head with the pulse sequence 90°(x)(^{1}H)-τ-90°(^{31}P)-180°(y)-90°(^{31}P)-τ-acq.[33] The 90° ^{1}H pulse width, supplied through the decoupling channel, was 28.5 μs, the ^{31}P 90° pulse length was 12 μs, and the delay τ 34 ms. Subtraction from data of an experiment without the ^{31}P pulses yielded a spectrum of only POC*H*-signals.

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