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Structural studies of the major polysaccharide in the cell wall of *Renibacterium salmoninarum*

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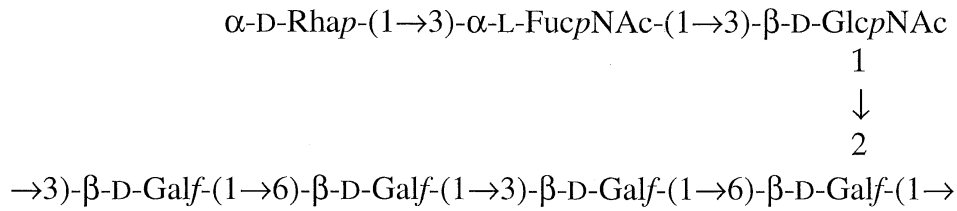
Abstract

The galactose-rich polysaccharide (GPS) in the cell wall of the Gram-positive bacterium *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD) of salmonids, has been studied by sugar and methylation analysis, partial acid hydrolysis, Smith degradation, FABMS, and ^{1}H and ^{13}C NMR spectroscopy. The data show that the GPS has a heptasaccharide repeating unit with the following structure:



Keywords: Cell wall polysaccharide; *Renibacterium salmoninarum*; Structure; NMR

1. Introduction

The Gram-positive bacterium *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), is one of the most important bacterial diseases of wild and cultured salmonids [1]. *R. salmoninarum* is a challenging pathogen because it survives and replicates within phagocytic cells and is transmitted both vertically and horizontally. Despite considerable efforts there exists no effective way to control the disease either through vaccination or treatment with antibacterial compounds. The immunodominant cell surface protein, p57, is assumed to be an important virulence factor of *R. salmoninarum* [2], but it is not a protective antigen [3].

Cell wall polysaccharides are considered important for the pathogenicity and immunogenicity of bacterial pathogens. Knowledge of the architecture of the protein-free cell wall skeleton of mycobacteria, another group of intracellular pathogens, has been central to the understanding of their physiology and pathogene-

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sis, and has also suggested new targets for chemotherapy of mycobacterial infectious agents [4]. Little is, however, known about the polysaccharides in the wall of *R. salmoninarum*. Previous studies have shown that its wall contains at least two polysaccharides; a unique peptidoglycan [5] and a galactose-rich polysaccharide (GPS) [5,6]. The GPS was found to constitute 60 to 70% of the dry weight of the protein-free wall preparations and is thus the quantitatively most important cell-wall polysaccharide of the bacterium [6]. The monosaccharide constituents of the GPS were determined to be galactose, rhamnose, *N*-acetylglucosamine, and *N*-acetylfucosamine [6]. The GPS appears to be covalently bound to the peptidoglycan through phosphodiester linkages, which may explain the resistance of *R. salmoninarum* cell walls to degradation by lysozyme and mutanolysin [6]. In the light of the chronic glomerulonephritis nature of BKD [7], it is interesting to note that lysozyme-resistant peptidoglycan–polysaccharide complexes derived from bacterial cell walls are commonly found to induce chronic inflammations and other pathological effects in animals [8]. Previous chemical studies of the GPS have not proceeded beyond analysis of the monosaccharide composition. In the present work we report on the structural elucidation of the GPS.

2. Experimental

Organism, growth conditions, and isolation of the galactose-rich polysaccharide (GPS).—A Norwegian isolate of *R. salmoninarum* was grown in liquid culture as described [9]. The GPS was isolated as previously described [6]. The GPS, extracted from cell walls with hot formamide (160 °C, 20 min), was freed from the extractant by extensive dialysis against tap water, lyophilized, and subsequently fractionated by gel filtration on a column (100×2.5 cm) of Sephacryl S-200. The column was eluted with Milli Q-water and fractions of 5 mL were collected. Calibration of the column was done with a mixture of blue dextran (void-volume), dextran (MW 9000) and glucose. Elution was monitored by a Knauer differential refractometer and fractions were assayed for neutral hexoses by the anthrone method [10]. Pooled fractions were analysed for monosaccharides by the alditol acetate analysis. In a typical run, 10 g cells (wet weight) originating from 4 L culture gave 20 mg (dry weight) purified GPS.

General methods.—Gas liquid chromatography (GLC) was performed on a HP-5890 instrument equipped with a flame-ionisation detector. Separation of alditol acetates was performed on an HP-5 fused-silica capillary column, using a temperature program: 200 °C for 5 min, then to 250 °C at 5 °C/min[1]. Separation of partially methylated alditol acetates was performed on a VG Tribrid gas-liquid chromatography–mass spectrometer (GLC–MS) instrument on a CP-Sil-8 CB-MS column, using a temperature program: 140 °C for 18 min, then to 250 °C at 20 °C/min.

NMR spectra were recorded for samples dissolved in D_2O at 70 °C with a Varian VXR 400 or Bruker DRX-600 instruments using standard pulse sequences. Chemical shifts are reported in ppm, using acetone (δ_H 2.225 and δ_C 31.07) as an internal reference. ^{1}H chemical shifts were obtained from 1D spectra when possible, or from cross-peaks in proton–proton correlated 2D spectra (COSY, relayed COSY, double relayed COSY). $J_{H1,H2}$-values were obtained from 1D or proton–proton correlated 2D spectra. Proton–carbon correlated spectra (HMQC) were obtained both with and without carbon decoupling. The $J_{H1,C1}$ values were obtained from a coupled HMQC experiment. Identification of inter-residue multiple bond correlations between anomeric protons and aglycone carbons was performed from an HMBC experiment.

FABMS spectra were recorded on a Jeol JMS-SX/SX-102A tandem mass spectrometer by bombardment of the samples, dissolved in a glycerol matrix, with Xe atoms of average translational energy of 6 keV. The matrix-assisted laser-desorption ionisation time-of-flight (MALDI-TOF) spectra were recorded on a Linear LDI 1700XP instrument, using 2,5-dihydroxybenzoic acid as matrix.

Partial acid hydrolysis.—In the first experiment the GPS (8 mg) was treated with 48% HF (1 mL) at 0 °C for 48 h, and the resulting oligosaccharides were isolated by gel filtration on a column (50×1.6 cm) of Bio-Gel P-2. The column was eluted with pyridine–acetate-buffer (0.1 M, pH 7.0, 6 mL/h), and fractions of 0.8 mL were collected. Several fractions were analysed by MALDI-TOF MS or FABMS. In the second experiment, the GPS (2.1 mg) was treated with 48% HF (0.25 mL) at 0 °C for 24 h, and the resulting oligosaccharides were reduced with $NaBD_4$ (10 mg in 0.5 mL 1 M NH_4OH) prior to separation on an Aminex HPX-42A size-exclusion HPLC-column (300×7.8 mm, Bio-Rad). The column was heated to 85 °C and eluted with Milli Q-water (0.5 mL/min), and fractions of 0.25 mL were collected. Calibration of the column was done with a mixture of

laminarin (void-volume), laminaripentamer, laminaritrimer, gentiobiose and glucose. Fractions in the heptamer to trimer region of the chromatogram were analysed by methylation analysis. Elution of oligosaccharides in both experiments were monitored by a Knauer differential refractometer.

Analytical methods.—Monosaccharide analysis of the GPS was performed by alditol acetate analysis as described [11]. The GPS was hydrolysed by 2 M CF_3COOH for 1 h at 121 °C. The monosaccharides were transformed to their corresponding alditol acetates and analysed by GLC. For quantification, external standards consisting of known quantities of reference monosaccharides were treated in the same manner as the samples. Methylation of the GPS and the oligosaccharides resulting from partial acid hydrolysis of the GPS was performed as described [12]. The methylated GPS was recovered using a Sep-Pak C-18 cartridge [13], while the methylated oligosaccharides were recovered using $CHCl_3$–water extraction [12]. The methylated GPS and oligosaccharides were hydrolysed as described [14], first by 5:75:20 CF_3COOH–HOAC–water for 2 h at 100 °C, and then by 1:4 CF_3COOH–water for 2 h at 100 °C. Prior to acetylation [11], the hydrolysis product from the methylated GPS was reduced with $NaBD_4$, while the hydrolysis products from the methylated oligosaccharides were reduced with $NaBH_4$. The partially methylated alditol acetates were analysed by GLC–MS as described above.

Smith degradation.—The GPS (6 mg) was treated with sodium periodate (43 mg) in NaOAc-buffer (2 mL, 0.1 M, pH 4.9) for 40 h at 4 °C in the dark. The excess of sodium periodate was destroyed by addition of ethylene glycol (40 μL), the product reduced with $NaBH_4$ (22 mg in 3 mL 1 M $NH_4$3H for 16 h), dialysed against tap water, and finally freeze-dried. The ^{1}H NMR spectrum showed that the oxidation was complete. The periodate-oxidized GPS was analysed by alditol acetate and methylation analysis. The product (4 mg) was treated with 0.5 M CF_3COOH (1 mL) for 19 h at 23 °C and for 30 min at 30 °C. The hydrolysis conditions were chosen based on preliminary experiments. The resulting oligosaccharide products were separated on Aminex HPX-42A as described above.

3. Results and discussion

Preparation of the GPS.—The GPS was extracted from the bacterial cell wall by extraction with hot formamide and separated from soluble peptidoglycan by gel filtration on Sephacryl S-200 as previously described [6]. In accordance with the previous study [6], the GPS eluted as a single symmetrical peak corresponding to a molecular mass of about 10,000 as determined against elution of dextran standards. The ^{1}H NMR analysis (below, Fig. 1) indicated that the polysaccharide preparation was pure.

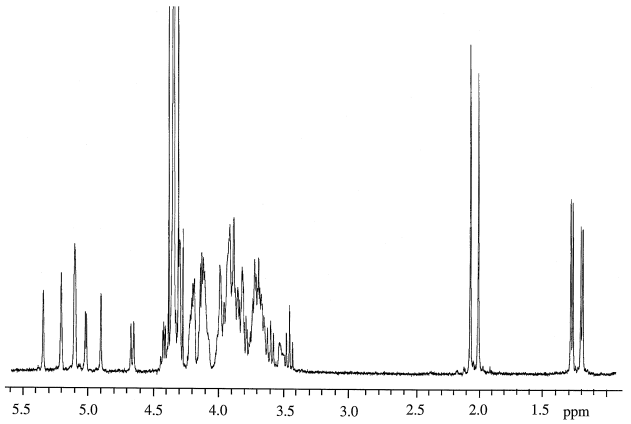


Fig. 1. 400 MHz ^{1}H NMR spectrum of the GPS from *R. salmoninarum.*

Monosaccharide constituents of the GPS.—Sugar analysis of the GPS (Table 1) indicated the presence of D-galactose, D-rhamnose, D-glucosamine and L-fucosamine in the relative proportions 5.4:1:1.4:1.1, which is in accordance with the 4:1:1:1 ratio suggested by Fiedler and Draxl [6]. The absolute configurations of the sugars were determined by GLC analysis of their acetylated (*S*)-2-butyl glycosides essentially as described by Gerwig et al. [15]. The absolute configuration of D-Rha was also confirmed by trimethylsilylation of the glycoside [15].

Identification of structural reporter resonances.—^{1}H NMR analysis of the GPS (Fig. 1) identified six signals for anomeric protons at δ 5.34, 5.20, 5.10, 5.02, 4.90, and 4.66 in a relative ratio of 1:1:2:1:1:1, indicating that the GPS has a heptasaccharide repeating unit. Two doublets were identified at δ 1.20 and

Table 1
Sugar analysis of the GPS from *R. salmoninarum*

Sugar	R_t^a (min)	Molar ratio	
		native GPS	periodate-oxidized GPS
Threitol	6.86	–	1
Rha	11.75	1	–
Ara	12.14	–	2
FucNAc	16.68	1.1	1.3
Gal	18.98	5.4	1.1
GlcNAc	25.80	1.4	0.9

[a]Retention time (min) on an HP-5 column as described in Section 2.

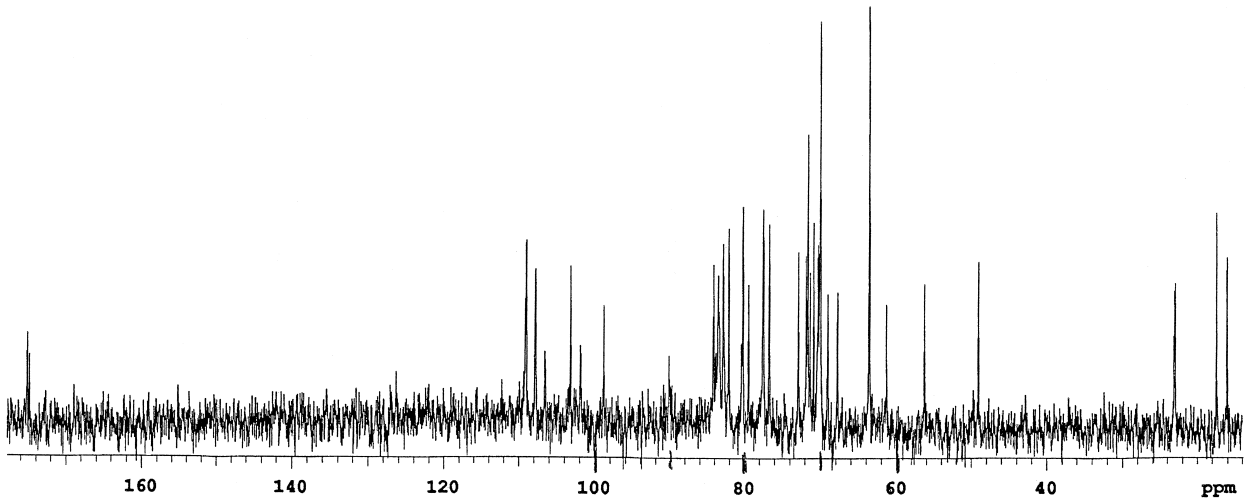


Fig. 2. 100.5 MHz ^{13}C NMR spectrum of the GPS from *R. salmoninarum.*

1.30 which were assigned to the methyl groups of the D-rhamnose and L-fucosamine residues. Furthermore, signals for two *N*-acetyl groups were observed at δ 2.02 and 2.08 which showed that the L-fucosamine and D-glucosamine residues were *N*-acetylated. ^{13}C NMR analysis of the GPS (Fig. 2) showed seven signals for anomeric carbons at δ 109.0, 108.9, 107.7, 106.4, 103.1, 101.8, and 98.8, showing that the GPS has a heptasaccharide repeating unit. The presence of four low-field signals ($\delta > 104$) in the anomeric region indicates that four of the monosaccharides are furanosidic. The identification of signals for two methyl carbons at δ 22.9, and two signals for carbonyl carbons at δ 175.1 and 174.8, further confirms that the amino sugars are *N*-acetylated.

Linkage analysis of the native GPS.—Methylation analysis of the GPS (Table 2) showed that all of the galactose residues were in the furanosidic form and that the other glycosyl residues were in the pyranosidic form. The residues were identified as 1,3-linked galactofuranose (Gal*f*), 1,6-linked Gal*f*, 1,2,6-linked Gal*f*, terminal rhamnopyranose (Rha*p*), 1,3-linked *N*-acetylglucosamine (Glc*p*NAc), and 1,3-linked 2-acetamido-2,6-dideoxygalactose (Fuc*p*NAc) with a relative detector response of 4.9:2.1:1.5:1.7:1.0:1.0. This indicates a molar ratio of 2:1:1:1:1:1 based on the deduced heptasaccharide repeating unit. Thus, methylation analysis indicates the presence of a branched structure in the GPS, where 1,2,6-Gal*f* is a branch point residue and where Rha*p* terminates the branch. In addition, low amounts of terminal Gal*f* was detected, assumed to represent the non-reducing terminal of the galactofuranose backbone.

Assignment of different spin-systems to the specific

Table 2
Methylation analysis of the GPS from *R. salmoninarum*

Methylated sugars[a]	Substitution	R_t^b (min)	Detector response (%)		
			native GPS	pentasaccharide[c]	periodate-oxidized GPS
2,3,4-Me_3-Rha*p*	terminal	17.46	0.14	0.11	–
1,2,4,5,6-Me_5-Gal*f*[d]	3-, reduced end	18.01	–	0.08	–
2,5-Me_2-Ara*f*	3	20.46	–	–	0.68
2,3,5,6-Me_3-Gal*f*	terminal	23.07	0.03	–	–
2,5,6-Me_3-Gal*f*	3-	24.09	0.40	–	–
3,5,6-Me_3-Gal*f*	2-	24.22	–	0.35	–
2,3,5-Me_3-Gal*f*	6-	24.26	0.17	–	–
3,5-Me_2-Gal*f*	2,6-	25.36	0.12	–	0.12
2,4-Me_2-Fuc*p*NAc	3-	26.11	0.08	0.27	0.1
2,4,6-Me_3-Glc*p*NAc	3-	27.03	0.08	0.19	0.1

[a] 2,3,4-Me_3-Rha*p* = 2,3,4-tri-*O*-methyl-rhamnopyranose etc.
[b] Retention time (min) on a CP-Sil-8 column as described in Section 2.
[c] Pentasaccharide isolated from Aminex HPX-42A after partial acid hydrolysis of the GPS as described in Section 2.
[d] Reduced by $NaBD_4$ at C-1.

Table 3
^{1}H and ^{13}C NMR chemical shifts at 70 °C for the GPS from *R. salmoninarum*

Atom		α-D-Rha*p*-(1→		→ 3)-α-L-Fuc*p*NAc-(1→		→ 3)-β-D-Glc*p*NAc-(1→		→ 6)-β-D-Gal*f*-(1→		→ 3)-β-D-Gal*f*-(1→		→ 6)-β-D-Gal*f*-(1→	
		A[a]		**B**		**C**		**D**		**E1/E2**		**F**	
H-1	C-1	4.90 (170)	103.1 (< 2)	5.02 (174)	98.8 (4)	4.66 (155)	101.8 (8)	5.34 (177)	106.4 (< 2)	5.10 (175)	108.9, 109.0 (< 2)	5.20 (174)	107.7 (< 2)
H-2	C-2	3.83	71.5[b]	4.31	49.9	3.90	55.9	4.21	90.1	4.29	80.0	4.14	82.3[b,c]
H-3	C-3	3.81	71.5[b]	3.94	77.6	3.71	79.8	4.10	76.8[b]	4.20	83.4	4.13	77.4[b]
H-4	C-4	3.45	72.8	3.90	71.8	3.60	68.9	3.94	83.6	4.14	82.9[c]	4.01	84.2
H-5	C-5	3.82	69.9	4.44	67.7	3.52	76.6	4.14[b]	70.3[b]	3.95	71.6[b]	4.01[b]	70.3[b]
H-6a, H-6b	C-6	1.30	17.5	1.20	16.2	3.81, 3.95	61.7	3.66[b], 3.88[b]	69.9	3.72–3.75	63.5	3.66[b], 3.88[b]	69.9
		Carbonyl group		Acetamido group									
			175.1	2.02	22.9								
			174.8	2.08	22.9								

[a]Values for $^{1}J_{C1,H1}$ and $^{3}J_{H1,H2}$ are given in parentheses.
[b]Approximate values.
[c]Assignments could be reversed.

sugar residues.—Using different 1D and 2D NMR experiments, most of the ^{1}H NMR resonances of the sugar residues (labelled **A**–**F**) in the GPS could be assigned, and the $J_{H1, H2}$ values could be determined. HMQC experiments allowed for the assignment of the corresponding C-signals and the $J_{C1,H1}$ values. This information together with published chemical shift data for sugars [16–18], allowed the assignment of the different spin-systems to the specific sugar residues (Table 3) and the determination of the anomeric configurations.

All ^{1}H and ^{13}C NMR signals of the spin-system of residue **A** were assigned. The $J_{C1,H1}$ value (170 Hz) indicates that **A** has an α-configuration. The small $J_{H1,H2}$ value (< 2 Hz) shows a *manno* configuration, and the fact that C-6 is a methyl carbon then establish that residue **A** is α-D-Rha*p*. The ^{1}H and ^{13}C NMR chemical shifts are in good agreement with reference values for α-Rha*p* [16] and thus support that residue **A** is located terminally.

All ^{1}H and ^{13}C NMR signals of the spin-system of residue **B** were assigned. The $J_{C1,H1}$ value (174 Hz) and the chemical shift of the H-1 signal (δ 5.02) indicate that residue **B** has an α-configuration. The chemical shift for the C-2 signal is 49.4, i.e., in the region for nitrogen-bearing carbons, and the signal for C-6 represents a methyl carbon (δ 16.2), which suggests that residue **B** is α-L-Fuc*p*NAc. The relative high numerical chemical shifts of the C-3 (δ 77.6) and H-3 (δ 3.94) signals, further confirm that residue **B** is 3-substituted.

All proton and carbon resonances of residue **C** were assigned. The chemical shift of the H-1 signal (δ 4.66), the $J_{H1,H2}$ value (8 Hz) and the $J_{C1,H1}$ value (155 Hz) suggest that residue **C** has the β-anomeric configuration. Residue **C** was identified as β-D-Glc*p*NAc based on the C-2 signal at δ 55.9, and signals for H-6a,b at δ 3.81 and 3.95. Compared with the corresponding chemical shifts for β-D-Glc*p*NAc, the C-3 (δ 79.8) and H-3 (δ 3.71) signals are shifted 5.0 ppm and 0.15 ppm downfield, respectively, indicating that **C** is 3-substituted.

The low-field C-1 (δ 106.4, 109.0, 108.9, and 107.7) and C-2 (δ 83.4, 80.0, 80.0 and 82.3) signals for residues **D**, **E1**, **E2**, and **F** indicate that they represent β-galactofuranosidic residues [18]. Due to intra-residual connectivities from the multiple bond correlation experiment (HMBC), assignments of C-4 (δ 83.6, 82.9, 82.9, and 84.2) and H-4 (δ 3.94, 4.14, 4.14, and 4.01) signals were possible. The C-3 signal for residue **E1** and **E2** (δ 83.4) was shifted 5.6 ppm downfield of the corresponding chemical shift for methyl β-D-galactofuranoside, and it was therefore concluded that residues **E1** and **E2** represent the two 3-substituted Gal*f* residues. The NMR signals from residue **E1** and **E2** have almost identical chemical shift values, indicating that they have similar surroundings in the GPS. The C-6 signal for the residues **D** and **F** was identical (δ 69.9) and was shifted 6 ppm downfield compared to the corresponding signal of methyl β-D-galactofuranoside. This suggests that both residue **D** and **F** are 6-substituted, and that they have similar surroundings in the GPS. Because the C-2 signal (δ 90.1) for residue **D** was shifted downfield (7.8 ppm) compared to that of residue **F** (δ 82.3), residue **D** was assumed to be 2-substituted and thus to represent the 2,6-disubstituted branchpoint β-D-Gal*f*. Residue **F** was assigned to the 6-substituted Gal*f*. Due to overlapping signals, full assignment of the ^{1}H and ^{13}C signals to the individual Gal*f* residues was not possible.

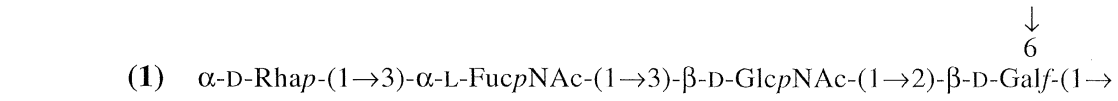
The combined results from sugar analysis, methylation analysis and ^{1}H and ^{13}C NMR spectroscopy therefore establish that the GPS has a heptasaccharide repeating unit, composed of terminal α-D-Rha*p* (residue **A**), 3-substituted α-L-Fuc*p*NAc (residue **B**), 3-substituted β-D-Glc*p*NAc (residue **C**), 2,6-disubstituted β-D-Gal*f* (residue **D**), two 3-substituted β-D-Gal*f* (residues **E1** and **E2**), and 6-substituted β-D-Gal*f* (residue **F**).

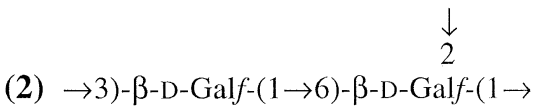
Identification of oligosaccharide fragments in the GPS by HMBC.—An HMBC experiment was conducted to obtain inter-residue connectivities between the anomeric protons and the aglycone carbons. Four cross-peaks representing inter-residue connectivities were identified (Table 4). In addition we observed

Table 4
Observed inter-residue multiple bond connectivities between some of the anomeric protons and aglycone carbons in the GPS from *R. salmoninarum*

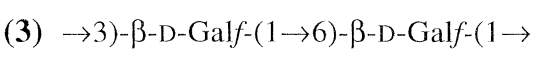
Anomeric proton	Aglycone carbon
4.90 (α-D-Rha*p*, **A**)	77.6 (C-3; → 3)-α-L-Fuc *p*NAc, **B**)
5.02 (α-L-Fuc*p*NAc, **B**)	79.8 (C-3; → 3)-β-D-Glc*p*NAc, **C**)
4.66 (β-D-Glc*p*NAc, **C**)	90.1 (C-2; → 2,6)-β-D-Gal *f*, **D**)
5.10 (β-D-Gal*f*, **E**)	69.9 (C-6; → 2,6)-β-D-Gal*f*, **D** and/or **F**)

several intra-residual connectivities. The data from the HMBC experiment indicated the presence of tetrasaccharide element (**1**) and disaccharide element (**2**) and/or (**3**):





and/or



Structural studies of oligosaccharides liberated from the GPS by partial acid hydrolysis.—Because galactofuranose is known to be involved in acid-labile glycosidic linkages [19], the GPS was incubated under mild acidic conditions in order to produce oligosaccharides. In the first experiment the resulting oligosaccharides were separated on a Bio-Gel P-2 column. MALDI-TOF analysis indicated that the major oligosaccharide was a pentasaccharide with $[M + Na]^+$ ion at *m/z* 901, indicating the presence of a pentasaccharide composed of one Rha, one FucNAc, one GlcNAc, and two Gal residues. In addition, we observed a trisaccharide, showing an $[M + Na]^+$ ion at *m/z* 593, indicating the presence of a trisaccharide composed of one FucNAc, one GlcNAc, and one Gal residue. A positive FABMS spectrum of the pentasaccharide showed as expected an $[M + H]^+$ ion at *m/z* 879, and the high-energy collision-induced fragmentation mass spectrum of this ion, using He as the collision gas, gave the fragmentation pattern shown in Fig. 3.

To obtain better resolution, the oligosaccharides in experiment 2 were reduced with $NaBD_4$ and separated on an Aminex HPX-42A column. Methylation

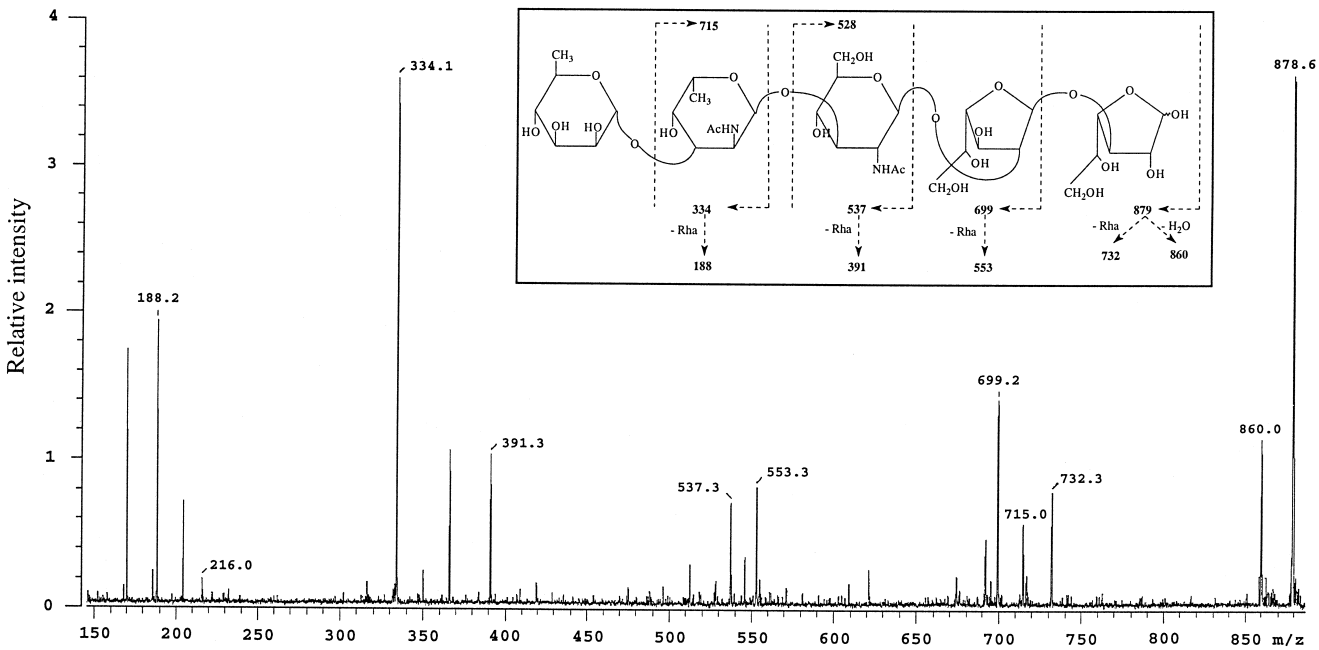


Fig. 3. Collision-induced fragmentation ions from a pentasaccharide, isolated after mild acid treatment of the GPS, obtained by positive FABMS. The molecular ion $[M + H]^+$ was observed at *m/z* 879, and the observed fragmentation pattern indicates the structure shown.

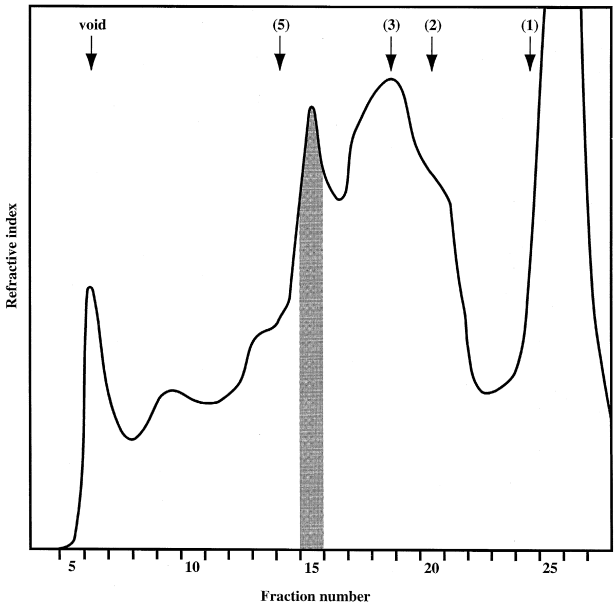
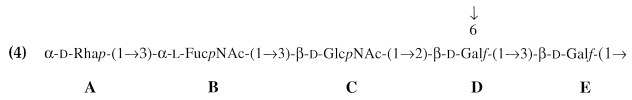


Fig. 4. Chromatography of the partially hydrolysed GPS on an Aminex HPX-42A column (300 × 7.8 mm, Bio-Rad), as detected by a refractive index monitor, of a mixture of oligosaccharides obtained by treatment of the GPS (2.1 mg) with 48% HF (0.25 mL) at 0 °C for 24 h. Fractions of 0.25 mL were collected. Elution of void, laminaripentamer (5), laminaritrimer (3), gentiobiose (2) and glucose (1) are shown with arrows. Methylation analysis showed that fraction 15 represented a relatively pure pentasaccharide.

analysis of a $NaBD_4$-reduced oligosaccharide isolated from the pentasaccharide region of the Aminex HPX-42A column (Fig. 4), identified terminal Rha*p*, 3-substituted galactitol-1*d*, 2-substituted Gal*f*, 3-substituted Fuc*p*NAc and 3-substituted Glc*p*NAc in the relative proportions of 1.4:1.0:4.4:3.4:2.4 (Table 2). The low amount of terminal Rha*p* and 3-substituted galactitol-1*d* may be explained by loss of the partially methylated alditol acetates residues due to their volatility. The finding of 3-substituted galactitol-1*d* indicated that this residue was the reducing end of the original oligosaccharide. The 2-substituted Gal*f* residue must originate from the branch point β-1,2,6-Gal*f*. On the basis of these analyses, the structure of the pentasaccharide element (**4**) was postulated:



From the above structure and the HMBC experiment, it can be concluded that the trisaccharide side chain is composed of α-D-Rha *p*-(1 → 3)-α-L-Fuc*p*NAc-(1 →

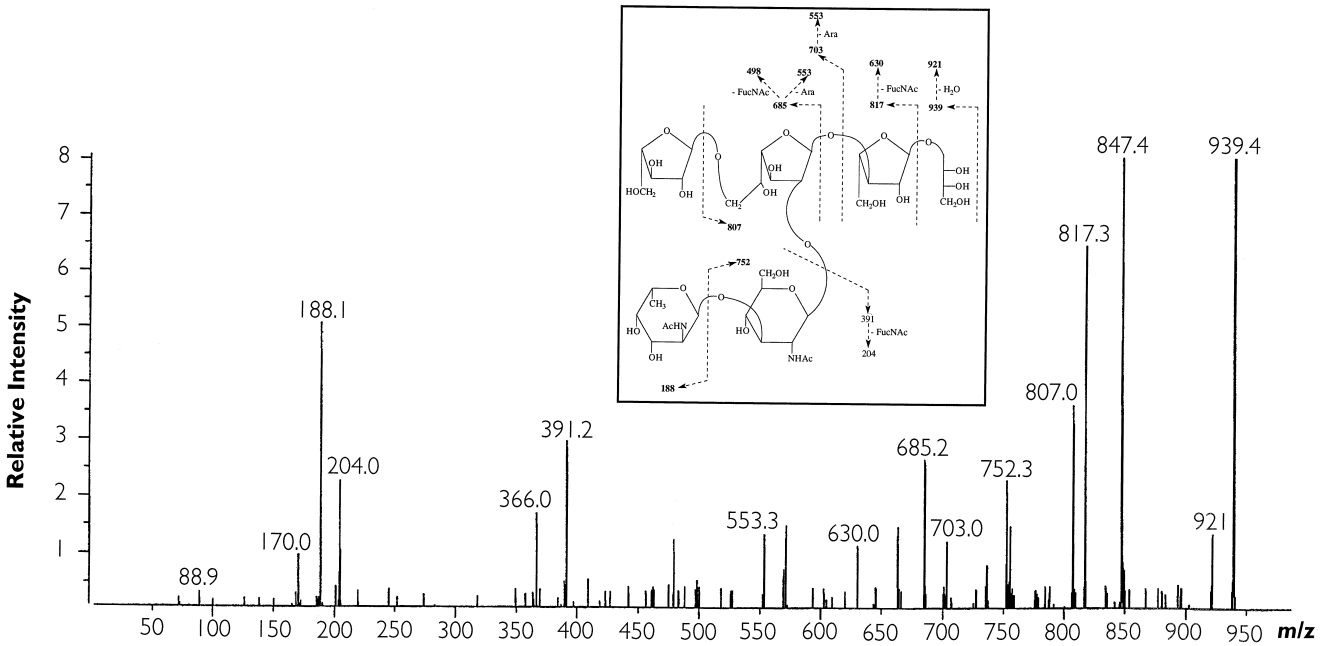
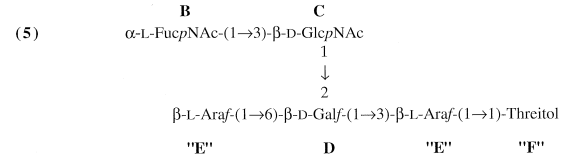


Fig. 5. Collision-induced fragmentation ions from a heptasaccharide, isolated after Smith degradation of the GPS, obtained by positive FABMS. The molecular ion $[M + H]^+$ was observed at m/z 939, and the observed fragmentation pattern indicates the structure shown.

3)-β-D-Glc*p*NAc. It can also be concluded that the side chain is linked to C-2 of the branch point β-1,2,6-Gal*f*, which is linked to the 3-substituted Gal*f*. The 3-substituted Gal*f* must therefore originate from the backbone of the GPS.

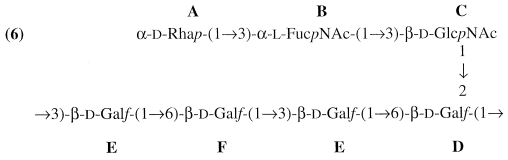
Structural studies of oligosaccharides liberated from the GPS by Smith degradation.—From the results of the methylation analysis, we deduced that the terminal α-D-Rha*p* and the 3- and 6-substituted Gal*f* residues would be sensitive to periodate oxidation. Therefore a Smith degradation of the GPS was performed, in order to produce oligosaccharides which overlapped the pentasaccharide, and subsequently deduce the positions of the four Gal*f* residues. A ^{1}H NMR spectrum of the periodate-oxidized material indicated a complete oxidation of the expected sugar residues. Sugar analysis of the periodate oxidized material resulted in identification of threitol, Ara, Gal, FucNAc, and GlcNAc in the molar ratio 1.0:2.0:1.1:1.3:0.9 (Table 1). Methylation analysis showed the presence of 3-substituted Ara*f*, 3-substituted Fuc*p*NAc, 3-substituted Glc*p*NAc, and 2,6-disubstituted Gal*f* with a relative detector response of 6.8:1.0:1.0:1.2 (Table 2).

Chromatography of the Smith-degraded GPS on an Aminex HPX-42A size exclusion column resulted in the appearance of several oligosaccharides, as could be predicted due to the presence of acid-labile linkages both from glycosides in the furanoid form and periodate-oxidised residues. A MALDI-TOF mass spectrum of one of the oligosaccharides showed an $[M + Na]^+$ ion at *m/z* 962 indicating a hexasaccharide composed of one FucNAc, one GlcNAc, two Ara, one Gal, and one threitol residue. The positive FABMS spectrum of the hexasaccharide showed an $[M + H]^+$ ion at *m/z* 939.4. The high-energy collision-induced fragmentation mass spectrum of this ion, using He as the collision gas, gave the fragmentation pattern shown in Fig. 5. The data therefore indicate the presence of the hexasaccharide element (**5**) in the Smith-degraded GPS:



In agreement with the previous data the results confirm that the side chain is composed of the trisaccharide element α-D-Rha *p*-(1 → 3)-α-L-Fuc*p*NAc-(1 → 3)-β-D-Glc*p*NAc-(1 → linked to the 2-position of the 2,6-disubstituted Gal*f* residue. Furthermore, the hexasaccharide element (**5**) also indicates that the other three galactofuranoses are present in the backbone of the GPS as indicated.

In conclusion the results of our studies therefore show the structure of the heptasaccharide repeating unit to be **6**:



The linear alternating 3- and 6-linked β-D-galactofuranosyl residues present in the backbone of the GPS may explain the identical chemical shift of C-6 of **D** and **F**, and C-3 of **E1** and **E2**, due to the similar surroundings of these residues. The molecular mass of the native GPS is about 10,000, and based on the molecular mass of the heptasaccharide repeating unit (1184), we deduce that the GPS is composed of approximately eight repeating units.

4. General discussion

Elucidation of the structure of the major polysaccharide in the cell wall of *R. salmoninarum* reveals several interesting molecular features. The GPS is shown to have a heptasaccharide repeating unit with a backbone consisting of linear alternating 3- and 6-linked β-D-galactofuranosyl residues and side chains consisting of a trisaccharide element containing *N*-acetyl-D-glucosamine, *N*-acetyl-L-fucosamine and D-rhamnose at the non-reducing end. To our knowledge the composition and sequence of monosaccharides in the GPS is unique to *R. salmoninarum.* The GPS is therefore not likely to show immunological cross-reaction with known (lipo)polysaccharides from other bacterial pathogens of salmonids, and may as such have a diagnostic value for identification of *R. salmoninarum.* Both D-rhamnose and *N*-acetyl-L-fucosamine are rare sugars in polysaccharides of Gram-positive bacteria.

Galactofuranose residues are quite common constituents of microbial polysaccharides although the glycosidic linkages involved vary. *Aspergillus* and *Penicillium* fungi contain immunologically active side chains of 1,5-linked β-D-galactofuranosides in their extracellular galactomannans [20]. Mycobacteria, well known intracellular pathogens of mammals, possess arabinogalactan as one of the major cell wall compo-

nents [21]. The galactan component of the mycobacterial arabinogalactan exists as a homopolysaccharide linked to a peptidoglycan and consists of linear alternating 5-linked α-D-Gal*f* and 6-linked β-D-Gal*f* residues. Similar to the GPS from *Renibacterium* [6], the mycobacterial galactan is covalently linked to the peptidoglycan through phosphodiester-linkages. Several *Arthrobacter* species also appear to contain galactofuranoses in their cell wall polysaccharides [22]. This is of particular interest since *R. salmoninarum* shares several other important features with *Arthrobacter*. Analysis of 16 S ribosomal RNA has shown that *R. salmoninarum* belongs to the *Arthrobacter–Micrococcus* subline of the actinomycetes [23]. In common with some *Arthrobacter* and *Micrococcus* species, *R. salmoninarum* possesses an αA3 peptidoglycan, has lysine as the diamino acid in the interpeptide bridge [5] and lacks mycolic acids [24].

Galactofuranoses, D-rhamnose and *N*-acetyl-L-fucosamine are rare, if not absent in vertebrates. This suggests that the GPS is antigenic in vertebrates and this is confirmed for rabbit by Fiedler and Draxl [6] and by ourselves (unpublished results). However, the GPS does not appear to be very immunogenic in Atlantic salmon (manuscript in preparation).

It has been shown that eggs of rainbow trout [25] and coho salmon [26] contain a lectin that selectively binds D-galactopyranose and L-rhamnose and which is suggested to have a role in the non-specific defence of the fish. Yousif et al. [26] and ourselves (unpublished results) were unable to demonstrate binding of the lectin to *R. salmoninarum* cells. The presented structure of the GPS, where the terminal rhamnose is in the D-configuration and galactose exists internally as furanosidic residues, could explain why this polysaccharide is not a ligand for the salmonid egg lectin(s).

Acknowledgements

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