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Carbohydrate Research 323 (2000) 235–239

CARBOHYDRATE RESEARCH

Note

Structure of the O-chain polysaccharide of the lipopolysaccharide of *Xanthomonas campestris* pv. *manihotis* GSPB 2755 and GSPB 2364

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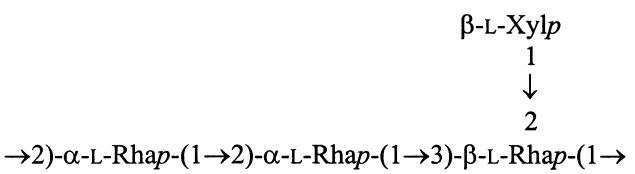
Received 2 August 1999; accepted 13 September 1999

Abstract

The O-chain polysaccharide of the lipopolysaccharide of *Xanthomonas campestris* pv. *manihotis* strains GSPB 2755 and GSPB 2364 was studied by sugar and methylation analyses and ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments. The polysaccharide was found to contain L-rhamnose and L-xylose in the ratio 3:1, and the following structure of the tetrasaccharide repeating unit was established:



Keywords: Phytopathogenic bacteria; *Xanthomonas campestris*; O-Chain polysaccharide; Lipopolysaccharide; Structure

Surface polysaccharides of the *Xanthomonas campestris* pathovars, including exopolysaccharides and lipopolysaccharides (LPSs), play an important role in the bacterium–plant interaction and are considered as bacterial virulence factors [1–3]. The elucidation of the LPS structure is necessary for understanding these processes on the molecular level and to answer the question whether the LPS may serve as a taxonomic character for differentation between *X. campestris* pathovars. However, data on the LPS structure of *X. campestris* are scarce ([4] and Refs. cited therein). Recently, we have established the structure of the O-

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PII: S0008-6215(99)00262-1

chain polysaccharide (OPS) of the LPS from *X. campestris* pv. *begoniae* GSPB 525 [4]. Now, we report the OPS structure of two strains of *X. campestris* pv. *manihotis*, GSPB 2755 and GSPB 2364.

The LPSs were isolated from bacterial cells of strains GSPB 2755 and GSPB 2364 by the phenol–water procedure [5] or by the phenol–chloroform–petrol ether extraction [6], respectively. High-molecular-mass OPSs were prepared by mild acid degradation of the LPSs followed by GPC on Sephadex G-50. Sugar analysis using GLC of acetylated alditol acetates [7] revealed rhamnose and xylose in the ratio $\sim$3:1 in both OPSs. GLC of acetylated (−)-2-octyl glycosides [8] showed that Rha and Xyl have the L configuration.

Methylation analysis of both OPSs revealed 2,3,4-tri-*O*-methylxylose, 3,4-di-*O*-methylrhamnose and 4-*O*-methylrhamnose in the ratios 0.5:2:1. Therefore, the OPSs are branched with terminal Xyl residues and 2,3-disubstituted Rha residues at the branching point, the remaining Rha residues being 2-substituted. These data showed also that all residues of both Xyl and Rha are in the pyranose form.

Like the sugar and methylation analyses data, the ^{1}H and ^{13}C NMR spectra of the OPSs from strains GSPB 2755 and GSPB 2364 were almost indistinguishable, and, hence, the OPSs from the two strains have the same composition and structure. Further NMR spectroscopic studies were performed with the OPS from strain GSPB 2755.

The ^{13}C NMR spectrum of the OPS (Fig. 1) contained signals for four anomeric carbons at δ 99.9–105.4, three CH_3–C groups (C-6 of Rha) at δ 17.8–18.1, one OCH_2–C group (C-5 of Xyl) at δ 66.4, and 15 other sugar ring carbons in the region δ 70.4–79.5. Accordingly, the ^{1}H NMR spectrum of the OPS contained signals for four anomeric protons at δ 4.56–5.21 and three CH_3–C groups (H-6 of Rha) at δ 1.28–1.34 (each 3H, d, $J_{5,6}$ $\sim$6 Hz). Therefore, the OPS has a tetrasaccharide repeating unit containing three residues of L-rhamnose and one residue of L-xylose.

The ^{1}H NMR spectrum of the OPS was completely assigned using 2D COSY and TOCSY experiments (Table 1). In the TOCSY spectrum, there were cross-peaks between H-1 and H-2,3,4,5_{eq},5_{ax} for Xyl and H-2,3,4,5 for two of the three Rha residues (Rha^{I} and Rha^{II}), but with only H-2 for Rha^{III}. However, cross-peaks between H-2 and H-3,4,5,6 and between H-6 and H-5,4,3,2 were present for all three Rha residues. Tracing connectivities in the COSY spectrum allowed the H-2,3,4,5 signals within each spin system to be distinguished.

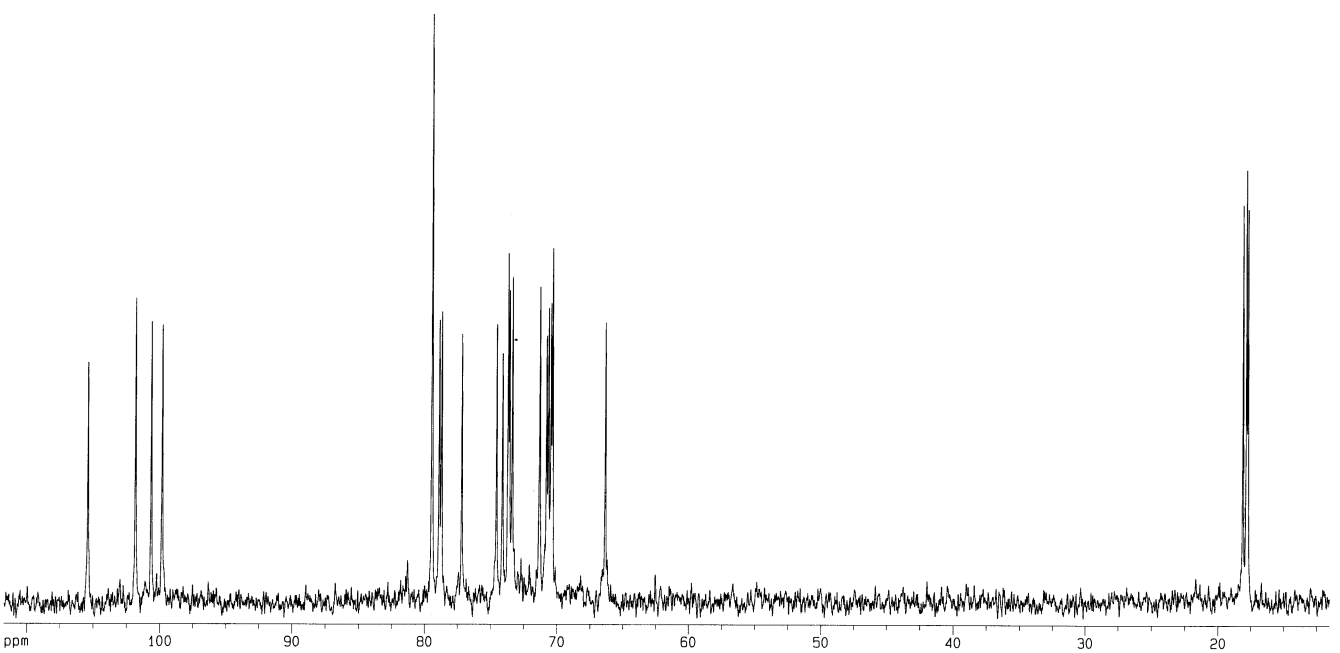

Fig. 1. ^{13}C NMR spectrum of the OPS from *X. campestris* pv. *manihotis* GSPB 2755.

Table 1
500-MHz ^{1}H NMR data of the OPS from *X. campestris* pv. *manihotis* GSPB 2755 (δ in ppm)

	H-1	H-2	H-3	H-4	H-5 (H-5$_{eq}$)	H-6 (H-5$_{ax}$)
→2)-α-L-Rha*p*^{I}-(1→	5.07	4.23	3.79	3.51	3.71	1.28
→2)-α-L-Rha*p*^{II}-(1→	5.21	4.06	3.93	3.50	3.94	1.30
→3)-β-L-Rha*p*^{III}-(1→ 2 ↑	4.84	4.17	3.69	3.47	3.46	1.34
β-L-Xyl*p*-(1→	4.56	3.39	3.43	3.65	4.01	3.24

Table 2
125-MHz ^{13}C NMR data of the OPS from *X. campestris* pv. *manihotis* GSPB 2755 (δ in ppm)

	C-1	C-2	C-3	C-4	C-5	C-6
→2)-α-L-Rha*p*^{I}-(1→	100.7	78.7	70.8	74.1 [a]	70.5	18.1
→2)-α-L-Rha*p*^{II}-(1→	101.9	79.5	71.3	73.7 [a]	70.4	17.9
→3)-β-L-Rha*p*^{III}-(1→ 2 ↑	99.9	79.5	78.9	73.6	73.4	17.8
β-L-Xyl*p*-(1→	105.4	74.6	77.2	70.7	66.4	

[a] Assignment could be interchanged.

As judged by the $J_{1,2}$ coupling constant value of 8.1 Hz, Xyl is β linked. The H-1 signals for the three Rha residues were broadened singlets, and, therefore, the configurations of the rhamnosidic linkages were determined using a NOESY experiment. The NOESY spectrum revealed intra-residue H-1,H-2 correlations for Rha^{I} and Rha^{II}, but H-1,H-3,5 correlations for Rha^{III} and Xyl. Hence, Rha^{I} and Rha^{II} are α linked, whereas Rha^{III} is β linked.

The NOESY spectrum of the OPS showed also strong inter-residue cross-peaks between the following transglycosidic protons: Rha^{I} H-1,Rha^{II} H-2 at δ 5.07/4.06, Rha^{II} H-1,Rha^{III} H-3 at δ 5.21/3.69, Rha^{III} H-1,Rha^{I} H-2 at δ 4.84/4.23, and Xyl H-1,Rha^{III} H-2 at δ 4.56/4.17. These data allowed determination of the modes of substitution and the full sequence of the monosaccharides in the repeating unit, as shown below. In addition, in the NOESY spectrum there were cross-peaks Rha^{I} H-1,Rha^{II} H-1 and Rha^{III} H-1,Rha^{I} H-1 at δ 5.07/5.21 (weak) and δ 4.84/5.07 (strong), respectively, which confirmed the (1→2) linkages between the corresponding sugar residues.

With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of the OPS was assigned using a H-detected ^{1}H,^{13}C HMQC experiment (Table 2). The chemical shifts of δ 70.4–70.5 for C-5 of Rha^{I} and Rha^{II} and δ 73.4 for C-5 of Rha^{III} confirmed the anomeric configurations determined by the NOE data (compare δ 70.0 and 73.2 in α-Rha*p* and β-Rha*p*, respectively [9]). Downfield displacements of the signals for C-2 of Rha^{I}, C-2 of Rha^{II}, and C-2,3 of Rha^{III} to δ 78.7–79.5 in the ^{13}C NMR spectrum of the OPS, as compared with their positions in the spectra of α-Rha*p* and β-Rha*p* at δ 71.3–74.0 [9], confirmed the substitution pattern of the OPS. In accordance with the lateral position of Xyl in the OPS, the chemical shifts for C-2,3,4,5 of this residue were close to those in β-Xyl*p* [10].

On the basis of these data, it was concluded that the OPS of *X. campestris* pv. *manihotis* GSPB 2755 and 2364 has the following structure:

β-L-Xyl*p*
1
↓
2
→2)-α-L-Rha*p*^{I}-(1→2)-α-L-Rha*p*^{II}-(1→3)-β-L-Rha*p*^{III}-(1→

The same sugar composition, but a different structure have been reported for the OPS of *X. campestris* pv. *begoniae* GSPB 525 [4], which, unlike the OPS studied in this work, lacks the strict regularity. The OPS of another xanthomonad, *X. campestris* pv. *campestris*, has a hexasaccharide repeating unit containing four α-L-Rha residues with additional Gal and GalA residues in the main chain [12].

Both strains of *X. campestris* pv. *manihotis* studied in this work were isolated in Nigeria from the natural host, *Manihot esculenta*. However, strain GSPB 2755 (StO3, IITA 243) was isolated in Onne in 1994, whereas strain GSPB 2364 (I1) one year earlier. When subjected to the Biolog identification method, an 'excellent identification' on the pathovar level to group II was achieved for strain GSPB 2755, whereas strain GSPB 2364 could be classified only by the genus to group III [11]. In addition, strains GSPB 2755 and GSPB 2364 differed in the fatty acid methyl ester (FAME) profile and belonged to FAME profile GC1 and GC1/GC2 groups, respectively [11]. The more remarkable is the identity of the OPS structure of both *X. campestris* pv. *manihotis* strains studied, which supports the view that the LPS of xanthomonads may be a useful taxonomic criterion on the pathovar level and may play a role in determining the narrow host specificity of the *X. campestris* pathovars [2].

1. Experimental

Growth of bacteria and digestion of cells.—*X. campestris* pv. *manihotis* strains GSPB 2755 and GSPB 2364 from the culture collection 'Göttinger Sammlung Phytopathogener Bakterien' (Göttingen, Germany) were cultivated in a 100-L fermenter at 28 °C on King's Medium B [13] with glycerol as carbon source. The cultures were stirred at 70 rpm at an aeration rate of 60 L/min. Cells were harvested at the late exponential phase by centrifugation, washed three times with 3 L EDTA–saline at 2 °C and lyophilized. Bacterial cells were extracted subsequently with ethanol for 2 h and acetone for 1.5 h to remove phospholipids, then sonicated for 10 min to disintegrate the cell walls. Nucleic acids were removed by digestion with DNAse and RNAse (5 mg/g lyophilized bacteria) for 8 h. Proteins were removed by digestion with proteinase K for 12 h. The resultant cell material was dialyzed against deionized water and lyophilized.

Isolation of LPSs and OPSs.—Bacterial cells of strain GSPB 2755 were suspended in deionized water at 70 °C, mixed (1:1) with warm aq 90% phenol (70 °C), and stirred for 30 min at 70 °C. The mixture was stored on ice for 12 h and centrifuged for 20 min at 17,000*g*. The aq phase was dialyzed against deionized water for 7 days and lyophilized. SDS-PAGE and staining according to the method of Tsai and Frasch [14] demonstrated the presence of S-type LPS.

LPS of strain GSPB 2364 was isolated by the method of Galanos et al. [6]. After washing, the bacterial mass was lyophilized, suspended in 250 mL warm aq 90% phenol (70 °C), stirred for 30 min at 70 °C and then overnight at ambient temperature. Chloroform (250 mL) and petrol ether (250 mL) were added, the suspension was stirred for 30 min at ambient temperature, and centrifuged (4 °C, 20 min, 9600 rpm). The pellet was extracted twice with a 2:5:8 phenol–$CHCl_3$–petrol ether (500 mL) mixture. After evaporation of solvents, the remaining solution was kept overnight in a cool room and the LPS was precipitated by adding water while the suspension was cooled on ice. After 1 h, the LPS precipitate was separated by centrifugation (4 °C, 10 min, 5000 rpm), washed three times with acetone, and dried in air.

The OPSs were prepared by degradation of the LPSs with aq 2% AcOH for 1.5 h at 100 °C followed by GPC on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate buffer (pH 4.5) as eluent and monitoring with a Knauer differential refractometer.

Sugar analysis.—The OPS (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h); monosaccharides were identified by GLC as their alditol acetates [7] using a Hewlett–Packard 5880 instrument with an Ultra 2 capillary column and a temperature gradient of 150 °C (1 min) to 290 °C at 10 °C/min. The

absolute configurations were determined by GLC of acetylated glycosides with (−)-2-octanol by the method [8] under the same chromatographic conditions as above.

Methylation analysis.—Methylation was carried out with methyl iodide in Me_2SO in the presence of solid NaOH [15]. Hydrolysis was performed as in sugar analysis; partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and a Nermag R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. Identification of partially methylated alditol acetates was performed using published data [16].

NMR spectroscopy.—OPS samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 50 °C. Data were acquired and processed using XWINNMR version 2.1 software. Mixing times of 200 and 100 ms were used in 2D TOCSY and NOESY experiments, respectively. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45).

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