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Carbohydrate Research 337 (2002) 1723–1728

CARBOHYDRATE RESEARCH

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Structures of the O-polysaccharide chains of the lipopolysaccharides of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* GSPB 271 and *X. campestris* pv. *malvacearum* GSPB 1386 and GSPB 2388

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Received 28 June 2002; accepted 6 August 2002

Abstract

O-polysaccharides of phytopathogenic bacteria *Xanthomonas campestris* were isolated by mild acid degradation of the lipopolysaccharides and studied by sugar and methylation analysis, along with ^{1}H and ^{13}C NMR spectroscopy. The following structures of the repeating units of the polysaccharides of *X. campestris* pv. *phaseoli* var. *fuscans* GSPB 271 (**1**) and *X. campestris* pv. *malvacearum* GSPB 1386 and GSPB 2388 (**2**) were established:

→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ **1**

α-D-Fuc*f*
1
↓
4
~65% OMe
|
3
→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ **2**

The O-polysaccharides of *X. campestris* are structurally similar to those of some *Pseudomonas syringae* strains.

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

1. Introduction

Phytopathogenic bacteria of the genus *Xanthomonas* are divided into a number of pathovars, each of which is characterised by a narrow and specific range of host plants.[1] The molecular mechanisms responsible for the narrow host specificity have not yet been unraveled. Specific structures of the outer-membrane lipopolysaccharides of phytopathogenic xanthomonads have been suggested to play a decisive role in host/parasite interactions.[2]

Recently, the structures of the O-specific polysaccharide chains (O-polysaccharides) of the lipopolysaccharides of *X. campestris* pvs. *begoniae* GSPB 525,[3] *manihotis* GSPB 2755 and GSPB 2364,[4] *vitians* 1839[5] and *vignicola* GSPB 2795 and GSPB 2796[6] have been established. Now, we report on the O-polysaccharide structures of *X. campestris* pvs. *phaseoli* var. *fuscans* GSPB 271 and *malvacearum* GSPB 1386 and GSPB 2388.

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00268-9

2. Results and discussion

X. campestris *pv.* phaseoli *var.* fuscans *GSPB 271.*—Sugar analysis of the O-polysaccharide using GLC of the alditol acetates and the acetylated glycosides with (−)-2-octanol showed that D-rhamnose is the sole O-polysaccharide component. Structural studies of the polysaccharide by one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy, including COSY, TOCSY, and ^{1}H, ^{13}C NMR experiments, showed that the polysaccharide has structure **1**. The specific optical rotation value of the polysaccharide, $[\alpha]_D + 75.0°$ (*c* 2, water), confirmed that rhamnose has the D configuration {compare published data,[7] $[\alpha]_D + 78.1°$ (water)}.

→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ **1**

This structure has been reported earlier for the O-polysaccharides of *Pseudomonas syringae*,[7,8] *Burkholderia cepacia*,[9] *Stenotrophomonas maltophilia*,[10] as well as of a common, lipopolysaccharide-associated antigen called A-band polysaccharide in *Pseudomonas aeruginosa* strains of different serotypes.[11–13]

X. campestris *pv.* malvacearum *GSPB 1386 and GSPB 2388.*—The ^{1}H and ^{13}C NMR spectra of the O-polysaccharides from both strains were indistinguishable, and structural studies were performed with the polysaccharide from strain GSPB 1386. Sugar analysis of the polysaccharide revealed 3-*O*-methylrhamnose, rhamnose, and fucose. The ratios of the monosaccharides were 0.5:3.0:1 and 0.4:2.1:1 (detector response) when hydrolysis was performed with 2 M CF_3CO_2H at 100 and 120 °C, respectively. These data indicated that 3-*O*-methylrhamnose and rhamnose are less stable towards acid than fucose. Determination of the absolute configurations of the monosaccharides by GLC of the acetylated (−)-2-octyl glycosides showed that both rhamnose and fucose are D. The D configuration of 3-*O*-methylrhamnose was determined by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see below).

Methylation analysis of the polysaccharide revealed 2,3,5-tri-*O*-methylfucose, 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, and 2-*O*-methylrhamnose in the ratios 1:0.9:1.5:1.2, respectively. When CD_3I was used for methylation, a ~2:1 mixture of 3-*O*-methyl-4-*O*-trideuteromethylrhamnose and 3,4-bis(*O*-trideuteromethyl)rhamnose was detected, whereas other sugar derivatives contained only CD_3-groups. Therefore, the polysaccharide is branched with terminal fucofuranose residues and 3,4-disubstituted rhamnose residues at the branching point. The remaining rhamnose residues are 2- and 3-substituted, and ~65% of the 2-substituted residues is 3-O-methylated.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for anomeric carbons at δ 101.9–103.0, CH_3–C groups (C-6 of Rha and Fuc) at δ 17.8–18.9, sugar ring carbons linked to oxygen in the region δ 69.3–85.8, and one O-methyl group at δ 58.0. The ^{1}H NMR spectrum of the polysaccharide (Fig. 2) contained, *inter alia*, signals for anomeric protons at δ 4.97–5.29, CH_3–C groups (H-6 of Rha and Fuc) at δ 1.20–1.38, and one O-methyl group at δ 3.47. The NMR spectra of the polysaccharide showed the lack of the strict regularity, most likely, owing to non-stoichiometric O-methylation.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). In the TOCSY spectrum, there were cross-peaks between H-1 and H-2–H-6 of Fuc*f* but only H-2 and H-3 of Rha*p* residues. The other ^{1}H NMR signals of rhamnose residues were assigned using correlations between H-6 and H-2–H-5 in the TOCSY spectrum and between

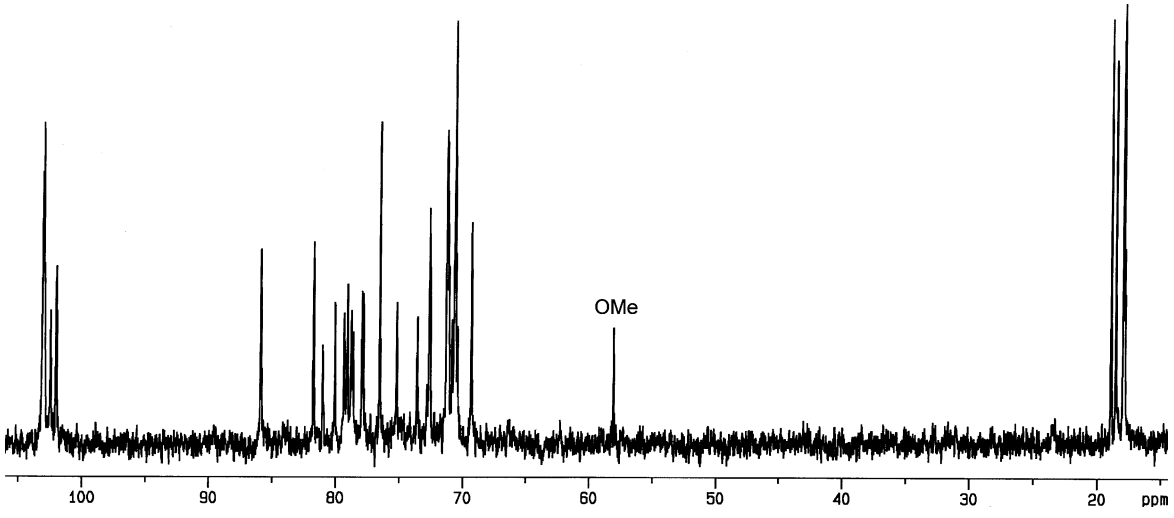

Fig. 1. ^{13}C NMR spectrum of the O-polysaccharide from *X. campestris* pv. *malvacearum* GSPB 1386.

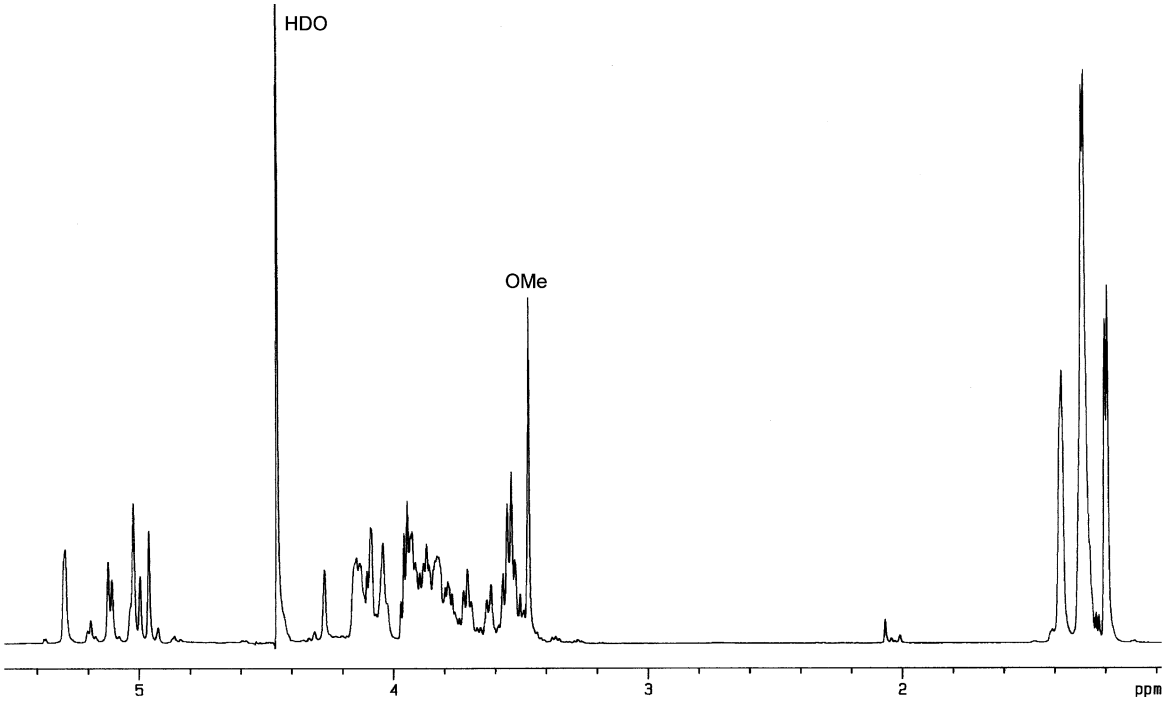

Fig. 2. ^{1}H NMR spectrum of the O-polysaccharide from *X. campestris* pv. *malvacearum* GSPB 1386.

coupled protons in the COSY spectrum. The spectra contained two series of signals that could be accounted for by partial O-methylation of one of the rhamnose residues (Rha*p*^{I}).

The ^{13}C NMR chemical shifts for C-2–C-6 of the terminal Fuc residue were typical of furanosides[14] and were close to the chemical shifts of α-fucofuranose.[8] As judged by the ^{13}C NMR chemical shifts, all Rha

Table 1
500-MHz ^{1}H NMR data of the O-polysaccharide from *X. campestris* pv. *malvacearum* GSPB 1386 (δ in ppm)

	H-1	H-2	H-3	H-4	H-5	H-6
O-Methylated repeating unit						
→2)-α-D-Rha*p*^{I}3Me-(1→	5.13	4.28	3.63	3.54	3.92	1.29
→3)-α-D-Rha*p*^{II}-(1→	4.97	4.09	3.83	3.56	3.79	1.29
→3)-α-D-Rha*p*^{III}-(1→ 4 ↑	5.03	4.15	4.04	3.71	3.93	1.38
α-D-Fuc*f*-(1→	5.29	4.11	3.96	3.54	3.87	1.20
Non-methylated repeating unit						
→2)-α-D-Rha*p*^{I}-(1→	5.11	4.05	3.94	3.53	3.87	1.29
→3)-α-D-Rha*p*^{II}-(1→	5.00	4.14	3.84	3.56	3.80	1.29
→3)-α-D-Rha*p*^{III}-(1→ 4 ↑	5.03	4.15	4.04	3.71	3.93	1.38
α-D-Fuc*f*-(1→	5.29	4.11	3.96	3.54	3.87	1.20

The chemical shift for MeO is δ 3.47.

Table 2
125-MHz ^{13}C NMR data of the O-polysaccharide from *X. campestris* pv. *malvacearum* GSPB 1386 (δ in ppm)

	C-1	C-2	C-3	C-4	C-5	C-6
O-Methylated repeating unit						
→2)-α-D-Rha*p*^{I}3Me-(1→	101.9	75.2	81.0	72.6[b]	70.8[c]	17.8[d]
→3)-α-D-Rha*p*^{II}-(1→	102.4	71.1[a]	79.0	72.6[b]	70.6[c]	17.9[d]
→3)-α-D-Rha*p*^{III}-(1→ 4 ↑	103.0	71.2[a]	81.7	78.7	69.3	18.5
α-D-Fuc*f*-(1→	102.9	77.9	76.5	85.8	70.6[c]	18.9
Non-methylated repeating unit						
→2)-α-D-Rha*p*^{I}-(1→	102.0	80.0	71.2	73.5	70.4[c]	17.9[d]
→3)-α-D-Rha*p*^{II}-(1→	102.9	71.2[a]	79.3	72.8[b]	70.6[c]	17.9[d]
→3)-α-D-Rha*p*^{III}-(1→ 4 ↑	103.0	71.2[a]	81.7	78.6	69.3	18.5
α-D-Fuc*f*-(1→	102.9	77.8	76.5	85.8	70.6[c]	18.9

[a–d]Assignment could be interchanged. The chemical shift for MeO is δ 58.0.

residues are in the pyranose form and are α-linked (e.g. compare δ 70.4–70.8 for Rha C-5 in the polysaccharide and δ 70.0 and 73.2 in α-Rha*p* and β-Rha*p*, respectively[15]). This conclusion was confirmed by intraresidue H-1,H-2 correlations revealed by the ROESY experiment.

Downfield displacements of the signals for C-3 of one of the non-methylated rhamnose residues (Rha^{II}) and C-3 and C-4 of another non-methylated residue (Rha^{III}) to δ 78.6–81.7, as compared with their positions in the spectra of α-Rha*p* at δ 71.3,[15] were observed in both major and minor series of the ^{13}C NMR spectrum of the polysaccharide and showed that Rha^{II} is 3-substituted and Rha^{III} is at the branching point and is 3,4-disubstituted. In the major series the signals for C-2 and C-3 of Rha^{I} were both shifted downfield to δ 75.2 and 81.0, respectively, whereas in the minor series shifted was only the signal for C-2 (to δ 80.0). Hence, all Rha^{I} residues are glycosylated at position 2 and most Rha^{I} residues are 3-O-methylated. In accordance with this finding, a ROESY experiment revealed a correlation between the signal for the O-methyl group and the H-3 signal of Rha*p*^{I} in the major series. Therefore, the polysaccharide has a tetrasaccharide repeating unit containing three residues of D-rhamnopyranose and one residue of D-fucofuranose, and in the most tetrasaccharide units one of the rhamnose residues is O-methylated. It remains unknown whether O-methylated and non-methylated units occur in the same polysaccharide chain or form two different polysaccharides.

The ROESY spectrum of the polysaccharide showed strong cross-peaks between the following anomeric and linkage protons in both series: Rha^{II} H-1,Rha^{I} H-2, Rha^{III} H-1,Rha^{II} H-3, Rha^{I} H-1,Rha^{III} H-3, and Fuc H-1,Rha^{III} H-4. These data defined the full monosaccharides sequence in the repeating units, which is shown below.

Similar effects of glycosylation in the α-Rha*p*^{I}3Me-(1→3)-α-Rha*p*^{III} and α-Rha*p*^{I}-(1→3)-α-Rha*p*^{III} disaccharide fragments (Table 2) indicated that 3-*O*-methylrhamnose has the same configuration as rhamnose, i.e. the D configuration.[16]

On the basis of the data obtained, it was concluded that the O-polysaccharide of *X. campestris* pv. *malvacearum* GSPB 1386 and GSPB 2388 has the following structure:

α-D-Fuc*f*
1
↓
4

~65% OMe
|
3

→3)-α-D-Rha*p*^{III}-(1→3)-α-D-Rha*p*^{II}-(1→2)-α-D-Rha*p*^{I}-(1→ **2**

A similar structure has been reported for the O-polysaccharide of *Pseudomonas syringae*,[8,17] which differs from the structure **2** in the absence of O-methyl groups and the location of Fuc*f* at position 4 of Rha*p*^{II} rather than Rha*p*^{III}.

3. Experimental

Growth of bacteria.—Bacteria *X. campestris* 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[18] 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 lyophilised. Bacterial cells were extracted subsequently with ethanol for 2 h and acetone for 1.5 h to remove phospholipids, and then sonicated for 10 min to disintegrate the cell walls. Nucleic acids were removed by digestion with DNAse and RNAse (5 mg per g lyophilised bacteria) for 8 h. Proteins were removed by digestion with proteinase K for 12 h. The resultant cell material was dialyzed against deionised water and lyophilised.

Isolation of lipopolysaccharides and polysaccharides.—Bacterial cells were suspended in deionised water at 70 °C, mixed (1:1) with warm aq 90% phenol (70 °C), and stirred for 30 min at 70 °C.[19] The mixture was stored on ice for 12 h and centrifuged for 20 min at 17,000*g*. The aqueous phase was dialyzed against deionized water for 7 days and lyophilized. SDS-PAGE and staining according to the method of Tsai and Frasch[20] demonstrated the presence of S-type lipopolysaccharides from both strains.

Polysaccharides were prepared by degradation of the lipopolysaccharides with aq 2% acetic acid for 1.5 h at 100 °C followed by GPC on a column (70 cm × 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 polysaccharide (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (100 or 120 °C, 2 h), monosaccharides were identified by GLC as the alditol acetates[21] using a Hewlett-Packard 5880 instrument with an DB-5 capillary column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The absolute configurations of rhamnose and fucose in the polysaccharide of *X. campestris* pv. *malvacearum* GSPB 1386 were determined by GLC of the acetylated glycosides with (−)-2-octanol[22] under the same chromatographic conditions as above.

Methylation analysis.—Methylation was carried out with CH_3I or CD_3I in dimethyl sulfoxide in the presence of methylsulfinylmethanide.[23] Hydrolysis was performed with 2 M CF_3CO_2H (100 or 120 °C, 2 h), the 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.

NMR spectroscopy.—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 XWIN-NMR 2.1 program. A mixing time of 120 and 200 ms was used in 2D TOCSY and ROESY experiments, respectively. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and external acetone (δ_C 31.45).

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