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Note

Structure of the O-polysaccharide of *Xanthomonas cassavae* GSPB 2437

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Abstract—The following structure of the O-polysaccharide of the phytopathogenic bacterium *Xanthomonas cassavae* GSPB 2437 was determined by sugar analysis along with ^{1}H and ^{13}C NMR spectroscopy:

β-L-Xyl*p*-(1→2)┐
→3)-β-D-Rha*p*-(1→3)-α-D-Rha*p*4NAc-(1→

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Keywords: Phytopathogenic bacteria; *Xanthomonas cassavae*; O-polysaccharide structure; Lipopolysaccharide

The Gram-negative bacterium *Xanthomonas cassavae*,[1] former *Xanthomonas campestris* pv. *cassavae*, causes leaf necrosis on cassava in East Africa. It produces an extracellular polysaccharide and a lipopolysaccharide, which appear to play a role in the susceptibility and the resistance response of the plant.[2] In various host–pathogen systems, the isolated lipopolysaccharides were shown to interact in a synergistic way to form a gel with pectic plant cell-wall polysaccharides from a host or a susceptible variety in vitro, whereas no interaction or exclusion occurred in case of a nonhost or a resistant variety.[3,4] Subtle differences in the lipopolysaccharide and especially in the often unique O-chain structure may be responsible for the highly specific interactions. Therefore, determination of the structure of the *X. cassavae* O-polysaccharide is of importance to further elucidate the host–pathogen interaction on the molecular level.

In this paper, we report on the structure of the O-polysaccharide of *X. cassavae* GSPB 2437, which was isolated from the lipopolysaccharide by mild acid degradation. Sugar analysis by GLC–MS of the alditol acetates[5] obtained after full acid hydrolysis of the polysaccharide revealed the presence of rhamnose, xylose and 4-amino-4,6-dideoxymannose (perosamine, Rha4N); the last sugar was identified by comparison with the authentic sample from the polysaccharide of *Citrobacter freundii* O9a,9b.[6] Determination of the absolute configurations of the monosaccharides by GLC of the acetylated (+)-2-octyl glycosides[7] showed that rhamnose has the D configuration and xylose the L configuration. The D configuration of Rha4N was established by the analysis of glycosylation effects in the ^{13}C NMR spectrum of the O-polysaccharide.[8]

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for three anomeric carbons at

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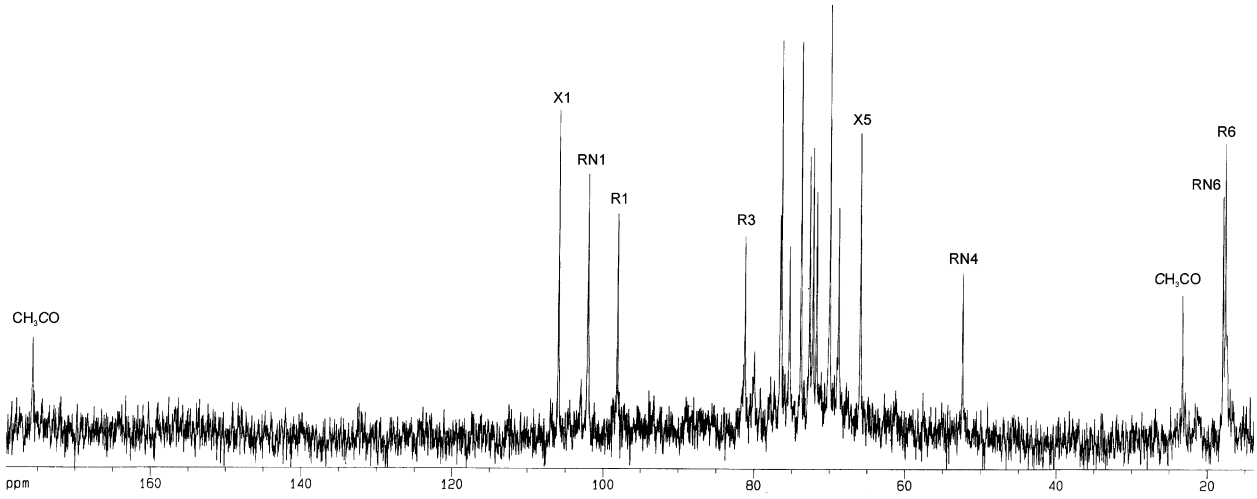

Figure 1. 125 MHz ^{13}C NMR spectrum of the O-polysaccharide. Arabic numerals refer to protons in sugar residues denoted as follows: R, Rha; RN, Rha4N; X, Xyl.

δ 98.2–106.0, two CH_3–C groups (C-6 of Rha and Rha4N) at δ 17.6 and 18.0, one CH_2–C group (C-5 of Xyl) at δ 66.0, one nitrogen-bearing carbon (C-4 of Rha4N) at δ 52.4, other sugar ring carbons in the region δ 69.0–81.4, and one N-acetyl group at δ 23.3 (Me) and 175.8 (CO). The ^{1}H NMR spectrum of the polysaccharide (Fig. 2) showed signals for three anomeric protons at δ 4.47–5.29, two CH_3–C groups (H-6 of Rha and Rha4N) at δ 1.24 and 1.33, other sugar protons in the region δ 3.28–4.29, and one N-acetyl group at δ 2.05. Therefore, the polysaccharide has a trisaccharide repeating unit containing one residue each of D-Rha, L-Xyl and D-Rha4NAc. The ^{1}H NMR spectrum contained also minor signals for a 6-deoxy sugar, which may belong to terminal monosaccharides of the main chain.

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 (Table 1). In the COSY spectrum, connectivities could be traced between all protons of the three sugar spin systems. The TOCSY spectrum showed correlation of H-1 with H-2,3,4,5a,5b for Xyl and H-1 with H-2,3,4,5,6 for Rha4NAc, whereas Rha was identified by correlation of H-1 with H-2 and H-6 with H-2,3,4,5. The spin systems for Rha4NAc was distinguished by correlation of proton at the nitrogen-

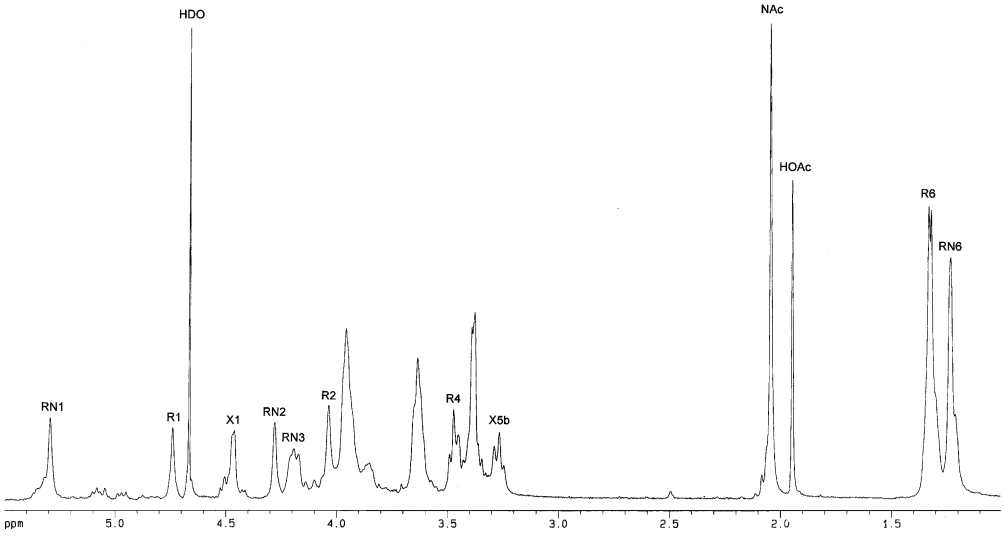

Figure 2. 500 MHz ^{1}H NMR spectrum of the O-polysaccharide. Arabic numerals refer to protons in sugar residues denoted as follows: R, Rha; RN, Rha4N; X, Xyl.

Table 1. 500 MHz ^{1}H and 125 MHz ^{13}C NMR data of the O-polysaccharide (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5(5a)	H-6(5b)
→3)-β-D-Rha*p*-(1→	4.74	4.04	3.65	3.47	3.40	1.33
→2,3)-α-D-Rha*p*4NAc-(1→	5.29	4.29	4.20	3.97	3.97	1.24
β-L-Xyl*p*-(1→	4.47	3.38	3.41	3.63	3.95	3.28
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Rha*p*-(1→	98.2	71.8	81.4	72.3	72.8	17.6
→2,3)-α-D-Rha*p*4NAc-(1→	102.2	76.7	75.4	52.4	69.0	18.0
β-L-Xyl*p*-(1→	106.0	73.9	76.5	70.1	66.0	

Signals for NAc are at δ_H 2.05, δ_C 23.3 (Me) and 175.8 (CO).

bearing carbon (H-4) with the corresponding carbon (C-4) at δ 3.97/52.4.

Comparison of the ^{13}C NMR chemical shifts of Xyl (Table 1) with those of the free monosaccharide indicated that Xyl in the polysaccharide occurs as β-linked pyranose. This was confirmed by the H-1 chemical shift of δ 4.47 and a relatively large $^3J_{1,2}$ coupling constant value (the exact value was difficult to determine owing to poor resolution of the H-1 signal and a coincidence of H-2 and H-3 resonances). As judged by the ^{13}C NMR chemical shifts, both Rha and RhaNAc are in the pyranose form, Rha is β-linked and Rha4NAc is α-linked (e.g. compare C-5 chemical shifts δ 72.8 for of Rha and δ 69.0 for Rha4NAc in the polysaccharide with δ 69.5 and 73.2 for α-Rha*p* and β-Rha*p*,[9] δ 69.5 and 71.9 for α-Rha4NAc and β-Rha4NAc,[10] respectively). The configurations of the glycosidic linkages were confirmed by an intraresidue H-1,H-2 cross-peak for α-Rha*p*4NAc and H-1,H-3 and H-1,H-5 cross-peaks for β-Rha*p* and β-Xyl*p* in the ROESY spectrum of the polysaccharide.

The ^{13}C NMR chemical shifts for C-2,3,4,5 of Xyl in the polysaccharide were close to those of the nonsubstituted β-Xyl*p*[11] and demonstrated the terminal position of this sugar residue in the polysaccharide. Downfield displacements of the signals for C-3 of β-Rha, C-2 and C-3 of α-Rha4NAc in the ^{13}C NMR spectrum of the polysaccharide, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides,[9,10] showed that β-Rha is 3-substituted and α-Rha4NAc is at the branching point and is 2,3-disubstituted. The ROESY spectrum of the polysaccharide showed correlations between the following anomeric protons and protons at the linkage carbons: β-Rha H-1,α-Rha4NAc H-3, α-Rha4NAc H-1,β-Rha H-3, β-Xyl H-1,α-RhaNAc H-2. These data defined the monosaccharides sequence in the repeating unit.

Therefore, the O-polysaccharide of *X. cassavae* GSPB 2437 has the following structure:

β-L-Xyl*p*-(1→2)┐

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

1. Experimental

1.1. Cultivation of bacteria

X. cassavae GSPB 2437 (UPB 047, isolated by H. Maraite in Ruanda in 1978) was cultivated in a 100 L fermenter (Braun and Diessel Biotech, Model U 100, Melsungen, Germany) on modified King's medium B (ingredients per L: proteose peptone 10.0 g, sucrose 10.0 g, sodium gluconate 10.0 g, KH_2PO_4 1.5 g, $MgSO_4$ 1.5 g) to an optical density of 1.1 (mid-logarithmic phase). Bacteria were harvested by centrifugation at 10,000*g* for 20 min at 4 °C, the pellet was washed three times by centrifugation with 10 L soln containing 10 mmol L^{-1} EDTA·2 H_2O and 0.1% (w/v) NaCl, suspended in water and lyophilised.

1.2. Isolation of lipopolysaccharide and O-polysaccharide

The lipopolysaccharide was extracted by the method of Westphal and Jann.[12] A suspension of lyophilised cells in distilled water (15 mL g^{-1}) was warmed to 68 °C, mixed with an equal vol of pre-warmed (60 °C) aq 90% phenol and the mixture was incubated in a water bath for 15 min at 68 °C with frequent stirring. After cooling to 4 °C, the phases were separated by centrifugation at 10,000*g* for 20 min, the upper aq phase was carefully siphoned off, treated with RNAse and dialyzed against deionised water for 72 h at 4 °C. The retentate was lyophilised, the crude lipopolysaccharide dissolved in sterile water (5 mg·mL^{-1}), the solution centrifuged at 10,000*g* for 20 min to remove insoluble materials, freed from protein and DNA contaminations by treatment with proteinase K and DNAse and lyophilised.

The O-polysaccharide was prepared by degradation of the lipopolysaccharide with aq 2% AcOH for 1.5 h at 100 °C followed by GPC on a column (70×26 cm) of Sephadex G-50 using 0.05 M pyridinium acetate buffer pH 4.5 as eluent and monitoring with a Knauer differential refractometer.

1.3. Sugar analysis

The polysaccharide (0.5 mg) was hydrolysed with 2 M CF_3CO_2H (100 °C, 2 h), monosaccharides were identified by GLC–MS as the alditol acetates[5] using a Finnigan MAT ITD-700 mass spectrometer and a temperature gradient of 150 °C (1 min) to 280 °C at 5 °C·min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (+)-2-octanol[7] using a Hewlett–Packard 5880 instrument on a DB-5 column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

1.4. NMR spectroscopy

A sample of the polysaccharide was deuterium exchanged by freeze drying three times from D_2O and then examined in a soln of 99.96% D_2O. NMR spectra were recorded using a Bruker DRX-500 spectrometer at 35 °C. A mixing time of 200 and 100 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 acetone (δ_C 31.45).

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