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Structural elucidation of the O-chain of the lipopolysaccharide from *Xanthomonas campestris* strain 8004

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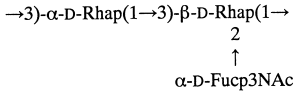
Abstract

A novel O-specific polysaccharide containing 3-acetamido-3-deoxy-α-D-fucose (Fuc3NAc) and D-rhamnose was isolated from the phenol-soluble lipopolysaccharide fraction of the plant associated bacterium *Xanthomonas campestris* strain 8004. The structure, determined by means of chemical analysis and 1D and 2D NMR spectroscopy, showed a branched trisaccharide repeating unit, as shown below:



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Lipopolysaccharides (LPSs) are ubiquitous, indispensible components of the cell surface of Gram-negative bacteria. As in bacterial pathogenesis in animals, LPSs apparently have diverse roles in bacterial pathogenesis of plants; these roles include protection of bacteria from antimicrobial compounds of plant origin, direct triggering of some plant defences and potentiation of triggering of specific plant defence responses.[1] Our laboratories are interested both in the structure of LPSs from phytopathogenic bacteria and in the relation of structure to the functions of LPS in plant–microbe interactions.[2,3] Much of our work on the effects of LPS on plants has used LPS derived from strain 8004 of *Xanthomonas campestris* pathovar *campestris*, a pathogen of cruciferous plants that can attack both cultivated brassicas and cruciferous weeds like *Arabidopsis thaliana*.[4]

Here we describe the novel structure of the O-antigen of LPS from strain 8004.

Freeze-dried bacterial cells were subjected to the sequential extraction procedure outlined in Section 1. The LPS fraction was found in the phenol phase deriving from hot phenol/water method. On SDS-PAGE electrophoresis, a ladder pattern typical of an LPS component was seen. A mild acetic acid hydrolysis allowed the removal of the lipid A moiety by precipitation leaving the polysaccharide moiety in solution. The compositional analysis of the O-chain *via* alditol acetate derivatives revealed the presence of two monosaccharides: rhamnose and 3-acetamido-3-deoxy-fucose (Fuc3N), both with D configuration. Rhamnose was present in the O-chain structure both as 3-substituted and 2,3-di-substituted pyranose residue while Fuc3N only as terminal pyranose residue, as inferred by methylation data.

Both ^{1}H and ^{13}C NMR spectra of O-chain (Figs. 1 and 2) suggested a regular structure built up of a

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trisaccharide repeating unit. In particular, the three anomeric proton signals at δ 5.252, 5.225 (d, $J = 3.2$ Hz) and 4.842 (indicated as **A**, **B** and **C**, respectively) were assigned to α-Rha*p*, α-Fuc*p* and a β-Rha*p*, respectively, on the basis of chemical shifts and $^{3}J_{H,H}$ values of anomeric proton signals.[5] The α anomeric configuration for **A** and **B** was established by a HSQC spectrum registered without decoupling during acquisition which showed values of 173 Hz and 171 Hz for the anomeric carbon signals at δ 101.4 and δ 100.1 which correlated to the signals at δ 5.252 and δ 5.225, respectively whereas the β anomeric configuration for **C** residue was indicated by the value of 162 Hz for the carbon signal at δ 97.8 correlating to the anomeric proton at δ 4.84.[6] A further indication of the β configuration of residue **C** was the observation of intraresidual nuclear Overhauser effect among H-1, H-3 and H-5 in the NOESY spectrum.

Starting from the proton anomeric signals **A**, **B** and **C**, COSY and TOCSY experiments allowed to establish the scalar connectivities among protons of each residue while by means of a HSQC spectrum the corresponding carbon signals were assigned (Table 1). The acetyl substitution at C-3 of residue **B** was proven by HMBC spectrum where both H-3**B** and the methyl signal at 2.08 ppm were correlated to a carbonyl signal at 175.6 ppm.

The low-field shifts of C-3**A**, C-2**C** and C-3**C** carbon signals with respect to the chemical shift values of the corresponding carbons of unsubstituted rhamnose confirmed the site of glycosylation of unit **A** and **C**.[7] As expected, the lacking of any downfield glycosylation shifts for the carbons of unit **B** confirmed its terminal location on the O-chain polysaccharide. The sequence of residues was deduced from the long-range heterocorrelated couplings measured for the anomeric protons of

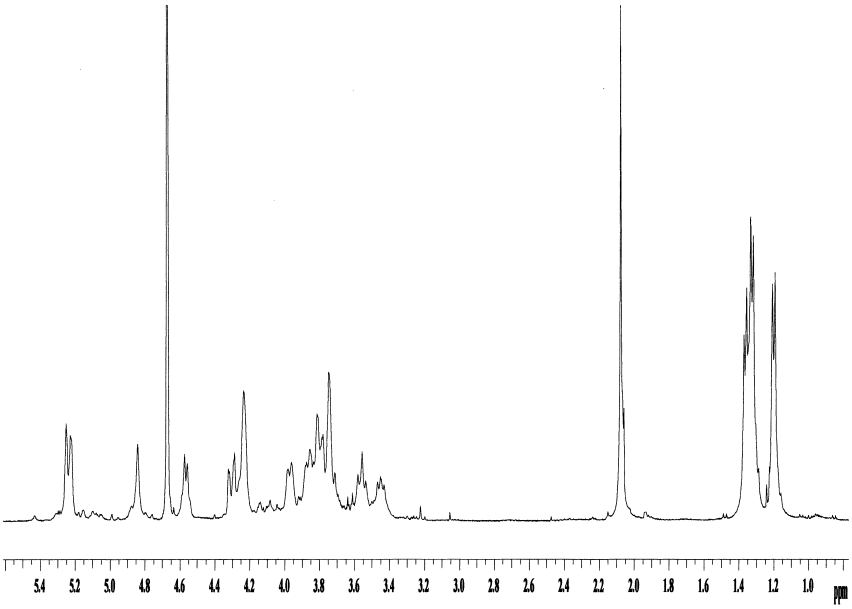


Fig. 1. The ^{1}H NMR spectrum of the O-chain of the LPS from *Xanthomonas campestris*.

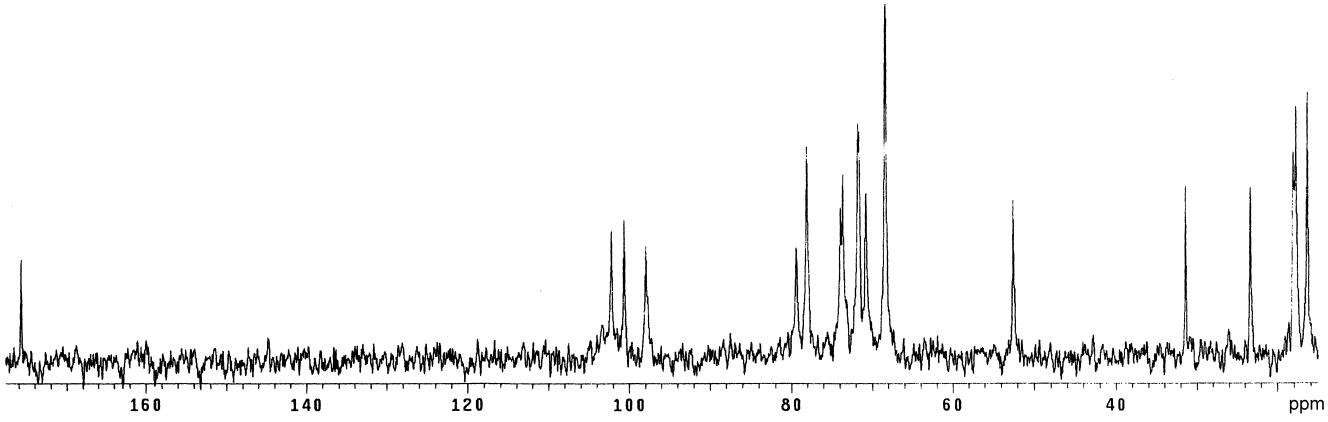


Fig. 2. The ^{13}C NMR spectrum of the O-chain of the LPS from *Xanthomonas campestris*.

Table 1
^{1}H and ^{13}C (*italic*) chemical shifts (ppm) of the O-specific polysaccharide

Sugar residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	5.252	4.233	3.960	3.552	3.809	1.328
3)-α-Rhap	*101.4*	*67.4*	*77.1*	*70.6*	*70.1*	*17.0*
B	5.225	3.787	4.310	3.740	4.567	1.190
t-α-Fucp3Nac	*100.1*	*67.7*	*51.8*	*71.2*	*67.6*	*15.6*
C	4.842	4.215	3.865	3.730	3.445	1.366
2,3-β-Rhap	*97.8*	*77.5*	*78.4*	*73.2*	*72.8*	*17.2*

The spectra are measured in D_2O at 303 K. Additional signals for the N-acetyl group are at 2.080/*23.7* ppm (methyl) and *175.6* ppm (carbonyl).

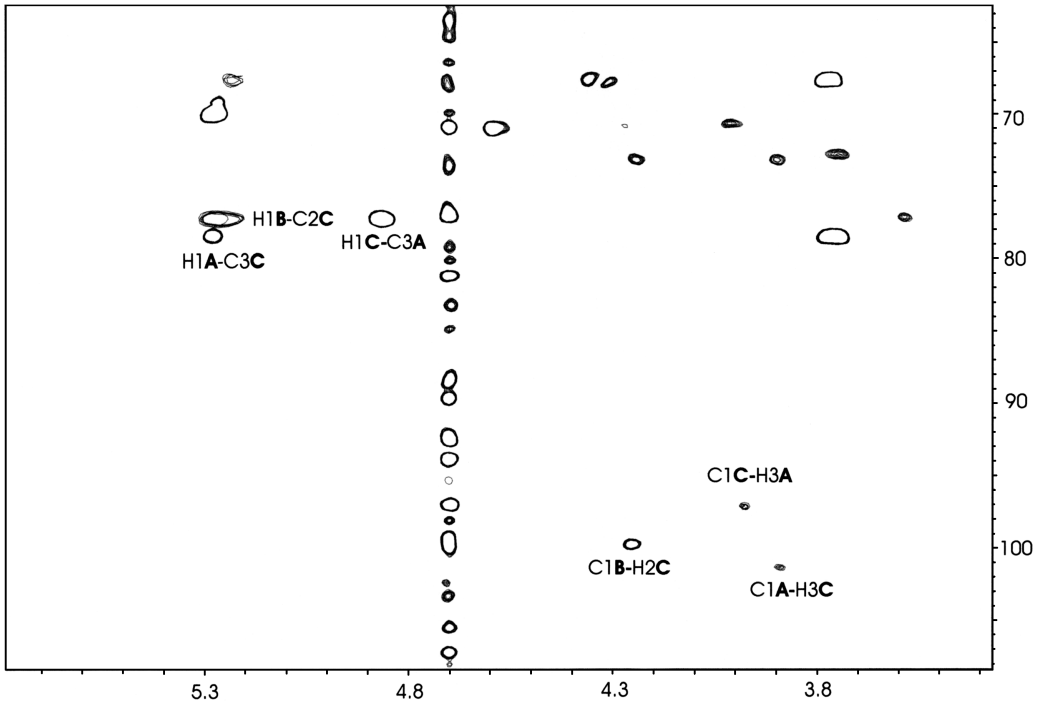
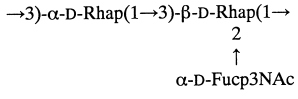


Fig. 3. The relevant HMBC correlations of the anomeric region of the O-chain are showed.

each residue by HMBC spectrum (Fig. 3). The inter-residual cross peaks between the proton anomeric signals 1**A**, 1**B** and 1**C** with the carbon signals of 3**C**, 2**C** and 3**A**, respectively, allowed the univocal establishment of repeating unit of the LPS structure from *Xanthomonas campestris* strain 8004.



Several kinds of repeating unit of LPS have been described in *Xanthomonas* species and most of them contain Fuc3NAc and Rha.[3] The structural arrangement of these residues in the O-antigen of strain 8004 that we report here is however novel, although a similar arrangement also occurs in strain 8183a (Prof. Y.A. Knirel, personal communication). Immunoanalysis of different pathovars within the species *X. campestris* (which attack different plant families) demonstrates considerable antigenic variation among LPSs, although some epitopes appear to be specific for certain pathovars.[4] These variations of LPS structure between strains of *X.campestris* are redolent of what is observed in bacterial pathogens of animals, although the significance of this for phytopathogenesis on different plants or plant families is as yet not known.

1. Experimental

1.1. Growth of bacteria, isolation of LPS and OPS

Strain 8004 of *Xanthomonas campestris* pv. *campestris* was grown in 100 litres of peptone-yeast extract-glycerol medium (NYGB) in a New Brunswick SSR 7 fermenter at 30 °C with aeration and stirring (150–500 rpm) to maintain pO_2 of dissolved oxygen at 10% saturation.[8] The seed culture was 500 ml of an overnight culture in NYGB grown to an OD at 600 nm of 1.0. After growth in the fermenter to an OD at 600 nm of 2.0, the culture was harvested by continuous centrifugation and washed with water. The cell mass was frozen at −80 °C and then freeze-dried. The dried cells (1.5 g) were extracted first with chloroform/petrol ether/phenol,[9] and then with hot phenol/water according to the conventional procedures.[10] The LPS content of the three fractions was checked by SDS/PAGE electrophoresis[11], Kdo[12] and 3-hydroxy fatty acid content and it was found in the phenol phase. This last was further purified from nucleic and protein material with enzymatic digestion with nuclease and protease to obtain pure LPS fraction (yield: 61 mg, 4.1% of bacterial dry mass). In order to obtain the O-polysaccharide chain, the LPS was hydrolysed with aq. 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant thus obtained (OPS fraction, 55 mg, 90% of LPS) was purified by gel permeation chromatography on a Sephacryl S300-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent and monitored with a Waters differential refractometer.

1.2. NMR spectroscopy

The ^1H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe, in the FT mode at 303 K. ^{13}C and ^1H chemical shifts are expressed in δ relative to methyl signal of acetone and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4), respectively. Two-dimensional spectra (DQF-COSY, TOCSY, NOESY, HSQC and HMBC) were measured using standard Bruker software. A mixing time of 200 ms was used in the NOESY experiment.

1.3. Gas chromatography

GC was performed on a Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), for compositional and methylation analyses the temperature program was: 150 °C for 5 min, then 5 °C min^{-1} to 300 °C, for absolute configuration analysis was: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min.

1.3.1. Compositional and methylation analysis. The monosaccharides were identified as acetylated *O*-methyl glycosides derivatives*:* briefly, samples were methanolysed with 2 M HCl/MeOH at 85 °C 20 h, dried under reduced pressure and then acetylated with acetic anhydride in pyridine at 80 °C 30 m. After work-up, the sample was analysed by GLC–MS. Absolute configuration of rhamnose was determined by GLC of acetylated glycosides of (+)-2-octanol according to the published method,[13] absolute configuration of Fuc3N was determined by the same method comparing it with an authentic sample obtained by *Xanthomonas campestris* pv. *vitians*.[14]

Methylation analysis was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide.[15] The hydrolysis of the methylated O-polysaccharide was carried out with 2 M TFA (120 °C, 1 h) and the partially methylated monosaccharides, reduced with $NaBD_4$, were converted in alditol acetates with acetic anhydride in pyridine at 80 °C for 30 min and analysed by GLC–MS.

Acknowledgements

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