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Note

Elucidation of two O-chain structures from the lipopolysaccharide fraction of *Agrobacterium tumefaciens* F/1

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Abstract—*Agrobacterium tumefaciens* F/1 produces two different O-chains, both are constituted of rhamnose and glucosamine: the less abundant has a linear disaccharidic repeating unit 3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→ and the second one 4)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→. The two intact antigenic moieties were studied in mixture by 2D NMR. Additional supporting data were obtained by periodate degradation, the major component was cleaved selectively, leading to a glucosamine glycoside, whereas the minor one was recovered unaffected.
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Keywords: *Agrobacterium tumefaciens*; O-chain; LPS; Structure

The Gram-negative bacteria belonging to the *Agrobacterium tumefaciens* species are object of very extensive investigation due to their potential biotechnological use. All the members of this family are phytopathogenic and induce the crown gall disease in most of dicotyledonous plants.[1]

Their mode of action implies the transfer and the integration of the T-DNA, a part of the tumour-inducing plasmid Ti, into the nuclear genome of the plant host cells. This peculiar feature makes them suitable tools for plant genetic engineering.

The lipopolysaccharides (LPSs) play an important role during the pathogenesis mechanism since they are involved in the adhesion process[2] on the host cell wall, committed step for the disease development. Despite the great interest in the biological aspects of these bacteria, few reports regarding their lipopolysaccharide (LPS) components have appeared so far.[3,4]

These macromolecules usually consist of an O-chain polysaccharide covalently linked to a core oligosaccharide, which, in turn, is linked to a lipid moiety (lipid A), and are located in the external membrane of the bacterium.

Pursuing our structural investigation on the LPSs of the *Agrobacterium* bacteria, now we report the structure determination of two O-chain moieties arising from the lipopolysaccharide fraction of *A. tumefaciens*, representative of group F/1.[5]

The LPS fraction of *A. tumefaciens* F/1 was isolated from dry cells according to the PCP method[6] and showed a ladder-like profile on SDS-PAGE electrophoresis in agreement with a S-type LPS (data not shown).

The GC–MS chromatogram of the *O*-acetylated methyl glycosides showed as main components rhamnose and glucosamine, besides minor amounts of mannose, galactose, glucose and Kdo; no heptose derivatives were detected.

Methylation analysis showed the presence of 3- and 4-substituted rhamnose residues and 3-linked 2-acetamido-2-deoxy-D-glucose only; minor signals were not investigated, being attributable to the oligosaccharide of the core LPS.

After mild acetic acid hydrolysis, the lipid A moiety was precipitated whereas the O-chain parts (PSs) were recovered from the supernatant liquor. The ^{1}H NMR spectrum of PSs showed (Fig. 1a) a rather simple

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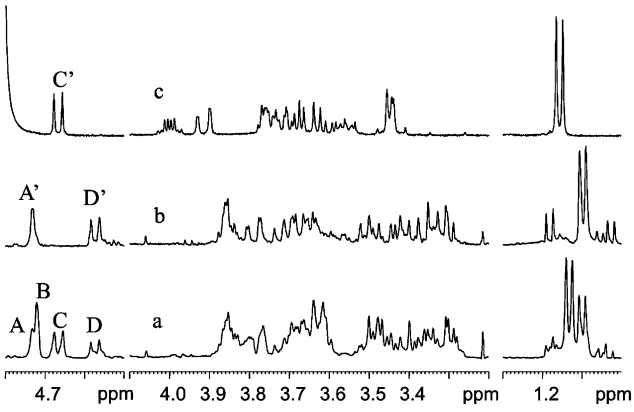

Figure 1. ^{1}H NMR spectra of (a) O-chain mixture, (b) minor O-chain survived to periodate treatment and (c) glucosamine glycoside **1** resulting from periodate treatment of the major O-chain component.

anomeric region, four intense signals were recognised, two unresolved doublets almost overlapped at 4.72 and 4.73 ppm, and two doublets at 4.67 and 4.57, respectively. In addition, the spectrum displayed two acetyl groups at 1.92 and 1.91, and two methyl groups at 1.13 and 1.10 ppm. The intensities of the four anomeric signals, appearing in the ratio of **A**:**B**:**C**:**D** = 1:1.5:1.5:1, strongly suggested the possibility of having a mixture of two regular polysaccharides, each consisting of a disaccharide repeating unit. In particular, the minor species was built from residues **A** and **D** and the more abundant from residues **B** and **C**.

The complete assignment of ^{1}H and ^{13}C (Fig. 2) signals was achieved (Table 1) combining the information from the DQ-COSY, TOCSY and HSQC 2D-NMR experiments. The residues **A** and **B** were identified as two different rhamnose units, the first substituted at O-3 and the second, more intense, linked at O-4, in agreement with the methylation data and the low-field glycosylation shift of the substituted carbon ^{13}C signals. As for the anomeric configuration of both **A** and **B** rhamnose residues, this was assigned to be α on the basis of the $^{1}J_{C1,H1}$ values (172.0 Hz) measured by a coupled gHSQC spectrum. In addition, the anomeric configuration of **A** residue, was further supported by the diagnostic chemical shift of carbon 5, whereas for rhamnose **B**, the chemical shift of carbon 5 was in agreement with an α configuration, upon consideration of the high-field β-glycosylation displacement effect.

Attribution of proton and carbon resonances of residues **C** and **D** led to their identification as two 2-acetamido-2-deoxy-glucose units, both β configured on the basis of their $^{3}J_{H1,H2}$ coupling constant (8.4 Hz), and both glycosylated at O-3, as suggested by the low-field displacement of C-3 signals.

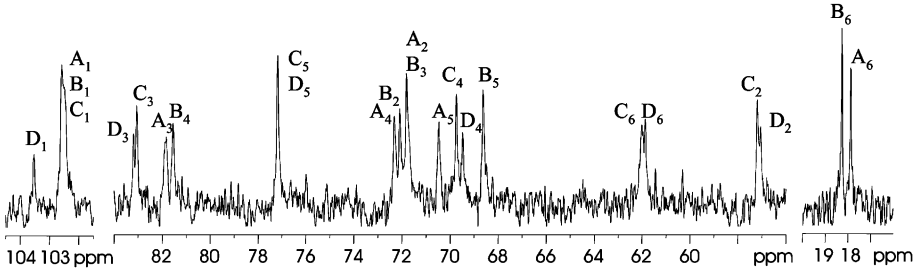

Figure 2. 75 MHz, 278 K ^{13}C NMR spectrum of the O-chain mixture produced by *A. tumefaciens* F/1, acetyl signals are coincident and omitted.

Table 1. ^{1}H (roman, 500 MHz, 323 K) and ^{13}C (italic, 75 MHz, 298 K) chemical shifts of major (residues **B** and **C**) and minor (residues **A** and **D**) O-chain components from *A. tumefaciens* F/1

Residue		1	2	3	4	5	6
3)-α-Rha*p*-(1→	**A**	4.73	3.87	3.65	3.35	3.87	1.10
		102.6	*71.8*	*81.8*	*72.3*	*70.5*	*17.9*
4)-α-Rha*p*-(1→	**B**	4.72	3.61	3.68	3.47	3.86	1.13
		102.6	*72.1*	*71.8*	*81.6*	*68.6*	*18.3*
3)-β-Glc*p*NAc-(1→	**C**	4.67	3.64	3.48	3.36	3.29	3.78–3.62
		102.4	*57.2*	*83.0*	*69.7*	*77.2*	*62.0*
3)-β-Glc*p*NAc-(1→	**D**	4.57	3.72	3.50	3.43	3.33	3.78–3.68
		103.6	*57.1*	*83.2*	*69.5*	*77.2*	*61.9*
Product **1**							
β-Glc*p*NAc-(1→	**C′**	4.67	3.70	3.56	3.44	3.44	3.92–3.75
		102.4	*57.5*	*74.9*	*71.1*	*76.5*	*62.4*
Aglycon		3.65	3.76	4.00	1.16		
		61.6	*85.0*	*68.4*	*18.0*		

Spectra of product **1** were recorded at 400 MHz, at 303 K. Chemical shifts of $NHCOCH_3$ groups: ^{1}H: 1.91; 1.92; ^{13}C: 23.6 and 175.9.

As for the absolute configuration, both rhamnose residues were L on the basis of the GC analysis of their acetylated 2-(+) octyl glycosides, whereas the N-acetylglucosamine residues were D as deduced by the analysis of their ^{13}C chemical shifts.[7] Actually the two possible diastereoisomeric disaccharides were considered: α-L-Rha*p*-(1 → 3)-β-L-Glc*p*NAc and α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc. Particularly relevant for the stereochemistry attribution was the comparison of the carbon chemical shifts of the C-4 signal of Glc*p*NAc residue versus the reference sugar. In the first case, a displacement of +0.9 ppm was expected differently from the high-field shift of −1.3 ppm diagnostic of the second disaccharide. The chemical shift of C-4 of both the **C** and **D** units (Table 1) occurred at higher fields than the value of the C-4 signal of the reference monosaccharide (β-Glc*p*NAc δ 71.2) then suggesting the D configuration for both Glc*p*NAc residues.

Conclusive structural information were obtained from the ROESY spectrum (Fig. 3). Actually the following dipolar couplings were recognised in the anomeric region: H-1 of **A** with H-3 of **D**, H-1 of **B** with H-3 of **C**, H-1 of **C** with H-4 of **B** and H-1 of **D** with H-3 of **A**.

These NOEs indicated the presence of two different polysaccharides, both constituted by a disaccharidic repeating unit, the minor component was built of residues **A** and **D** and possessed the following structure: 3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→; the major component instead was constituted from residues **B** and **C**, according to the following repeating unit: 4)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→.

In order to confirm the above results, the whole O-chain mixture was treated with sodium periodate,[8] this degradation protocol was chosen due to its high selectivity being only the carbon–carbon linkages of vicinal hydroxyl groups to be cleaved oxidatively. In the present case the only periodate sensitive residue was rhamnose **B**, belonging to the major O-chain component.

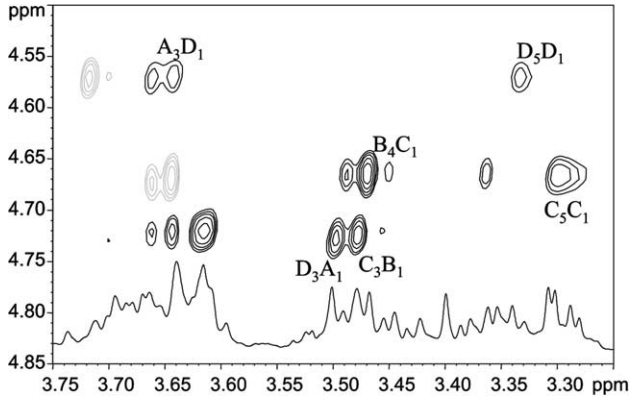

Figure 3. 500 MHz, 323 K, expansion of ROESY spectrum measured on the O-chain mixture produced from *A. tumefaciens* F/1. NOE effects are displayed with black lines, artefacts due to TOCSY effects are in grey.

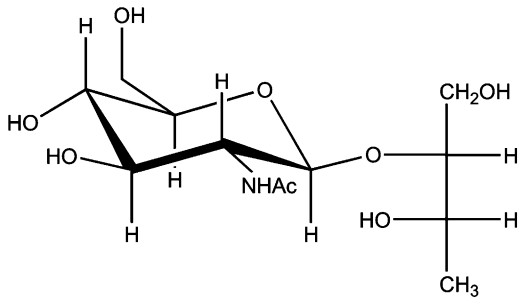

Figure 4. Structure of the glucosamine glycoside obtained by periodate degradation of the major O-chain.

Accordingly, from the gel-chromatography of the crude reaction, two main fractions were obtained. The first eluted one was the minor polysaccharide component of the original mixture, actually the proton spectrum showed two signals in the anomeric region (Fig. 1b) coincident with those formerly labelled as **A** and **D** in agreement with the structure previously assigned 3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→. The second more retained fraction (proton spectrum in Fig. 1c) displayed only one proton in the anomeric region at the same chemical shift of the original **C** residue, analysis of its COSY and gHSQC spectra (Table 1) proved that it was the expected degradation product **1** (Fig. 4) descending from the major O-chain component.

The obtaining of a polysaccharide and the glycoside **1** from the periodate degradation strongly suggested a mixture of two O-chain components of which only one was labile to oxidation. However, the possibility to have a single O-chain polysaccharide with a blockwise architecture alternating fragments of: [3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→]$_n$ and [4)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→]$_n$ repeating units, could not be ruled out at this stage.

Supporting evidences to the presence of two different antigenic moieties, were provided by the SEC profiles of both the native O-chain mixture and the Smith-degraded polysaccharide. According to the HPLC chromatograms (Fig. 5), periodate degradation did not induce a drastic molecular weight decrease of the polysaccharide component (Fig. 5b), with respect to the native one. Such decrease could reasonably be related to the chemical treatments, and not to the presence of blocks of [3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→]$_n$ motifs in a single polymer chain. The minor peak (Fig. 5b, 100 kDa) might be connected to byproducts appearing in the proton spectrum (Fig. 1b) in the methyl signals' region.

In conclusion, the above data suggest that *A. tumefaciens* F/1 is an S-type bacterium. Its LPS fraction has a high molecular weight and presents two linear O-chain moieties both with a disaccharidic repeating unit. The minor one, which accounts for approximately 40% of the whole mixture, has the following repeating

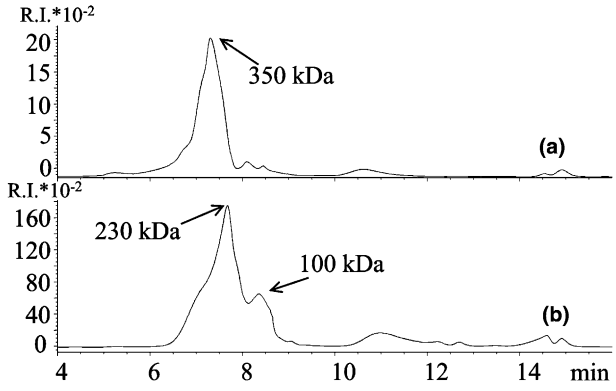

Figure 5. Refractive index chromatograms of HPLC purification on TSK G3000 PW_{XL} of: (a) O-chain mixture, (b) periodate degraded O-chain. Both samples were already purified on Sephacryl HR-100.

unit: 3)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→. The only difference with the major one lies in the substitution pattern of the rhamnose unit that is 4-*O* linked, the following repeating unit is determined for the major component: 4)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1→.

These two O-chain structures are not new and their chemical and spectroscopical data are in agreement with those reported for *Serratia marcescens* serogroup O17 and O19 (for the minor component),[13] and serogroup O1 (for the major component).[14]

It could be surprisingly that this bacterium shares the same repeating unit motifs of the genetically distant bacterium *S. marcescens*, whereas the same does not happen within members of the same species. Actually the two repeating units reported so far are quite different and are: 3)-α-D-Ara*f*-(1 → 3)-α-L-Fuc*p*-(1→,[3] and 3)-α-L-6dTal*p*-(1→ randomly acetylated at C-4,[4] for groups B6 and C58, respectively.

The structural diversity among these *A. tumefaciens* species might be a feature of biological relevance when considering the role played by the LPS during the bacterium-plant interaction. Probably, among these three bacteria there is not a direct competition on the plant cell wall, since they display different antigen, and in *A. tumefaciens* F/1, the exposition of more than one antigenic moiety might enhance the probability of adhesion[2] and consequently the success of the infection.

1. Experimental

A. tumefaciens strain DSM 30206, representative of the F/1 group, was grown at 27 °C in liquid shake culture (200 rpm) in Nutrient Broth (DIFCO). Cells were pelleted at 2000*g*, washed with distilled water and freeze–dried.

Dried cells (4.3 g) were extracted according to the PCP method[6] yielding to the LPS fraction (4.1% g_{LPS}/$g_{dry\ cells}$), used without further purification steps.

Discontinuous SDS-PAGE (sodium dodecyl sulfate polyacrylamide electrophoresis),[9] was performed with 12% separating gel on a miniprotean gel system from Bio-Rad; the samples where run at constant voltage (150 V) and stained according to the procedure of Kittelberger and Hilbink.[10]

1.1. Chemical analysis of LPS

Monosaccharides were analysed as their acetylated methyl glycosides derivatives.[11] Methylation procedure and alditol acetylated derivatives were obtained as described.[12]

1.2. Isolation of the O-specific polysaccharide

The LPS in 1% AcOH (10 mg/mL) was hydrolysed at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000*g* for 20 min and the clear supernatant freeze–dried and purified by SEC on Sephacryl HR-100 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow 0.4 mL/min). Column eluate was monitored with a R.I. refractometer (K-2310 Knauer); O-chain was eluted as major peak close to the void volume (31% yield from LPS).

1.3. Smith degradation product of O-chain

Periodate degraded product was obtained from O-chain according to the procedure reported in literature[8] and purified as reported above for the O-specific polysaccharide fraction.

1.4. Molecular weights determination

HPLC analysis were performed on a Agilent 1100 binary pump equipped with a refractive index detector; samples (20 μL each, 1 mg/mL native O-chain mixture, 5 mg/mL minor O-chain component from periodate degradation) were run on a on TSK G3000 PW_{XL} (Tosoh Biosciences, flow 0.7 mL/min, eluent: water). Both samples were already purified on Sephacryl HR-100. Molecular weights calibration was performed with Dextran Standards run in the same conditions.

1.5. NMR spectra acquisition

NMR experiments on the periodate degraded products were carried out at 323 K for the minor O-chain component and at 303 K for product **1**, on a Bruker DRX 400 equipped with a *z*-gradients multinuclear reverse probe, spectra on the O-chain mixture were measured at 323 K on a Varian Inova 500 of Consortium INCA (L488/92, Cluster 11), equipped with a *z*-gradients reverse probe.

Chemical shift of spectra recorded in D_2O are expressed in ppm relative to internal acetone ($^1H = 2.225$ and $^{13}C = 31.5$ ppm). Two-dimensional spectra (DQ-COSY, TOCSY, ROESY gradient-HSQC) were measured using standard manufacturer software.

For the homonuclear experiment, 512 FIDs of 2048 complex data points were collected, with 40 scans per FID. The spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak. For the HSQC spectrum, 256 FIDS of 2048 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Conversion of the Varian data and processing was performed with standard Bruker Xwinnmr 1.3 program, the spectra were assigned using either Xwinnmr or the computer program PRONTO.[15]

^{13}C spectrum were acquired on a Varian Gemini 300 (operating frequency for ^{13}C spectra: 75 MHz), equipped with a dual probe at a temperature of 25 °C.

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