Characterisation of the α-(1→3) Homopolymer of L-*glycero*-D-*manno*-Heptose Units Isolated from the O-Chain Polysaccharide of *Agrobacterium radiobacter*

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This paper provides the first report of the molecular primary structure of the O-chain produced from *A. radiobacter* group M2–1. The repeating unit was determined, by means of chemical and spectroscopical analyses, to have the following structure: [3)-α-L-*glycero*-D-*manno*-heptose-(1→]$_n$. The relevant feature of this structure is represented by the particular residue involved: L-*glycero*-D-*manno*-heptose. This monosaccharide is almost ubiquitous in Gram-negative bacteria, but it is exclusively located in the Core region and only in one other case has it is reported as an O-chain constituent. The knowledge of the chemical structure of the O-chain of *A. radiobacter* will be useful for comprehending the bacterium-host recognition mechanism at the molecular level.

Introduction

Agrobacterium radiobacter is a soil inhabiting Gram-negative bacterium, and it is genetically related to *Agrobacterium tumefaciens*, a bacterium of the same genus responsible for crown gall plant disease.[1]

These two bacterial species possess many common features and both belong to the same cluster, as has been proved by the chromosomal genome homology determined by thermal DNA hybrid stability experiments.[2]

Despite the several similarities existing amongst *A. tumefaciens* and *A. radiobacter*, the latter is not a phytopathogenic bacterium at all, and it gains its importance as a biocontrol agent because it can protect plants from the action of *A. tumefaciens*.

To recognise the importance of *A. radiobacter*, the mechanism of action of *A. tumefaciens* must be considered. This bacterium is responsible for crown gall disease in a wide range of dicotyledonous plants; it transfers the T-DNA, a portion of its plasmidial DNA (called "Ti", i.e., tumour-inducing), into the plant host genome, where it is integrated, causing the uncontrolled growth of the modified plant cells and then the formation of the tumour.

The development of the pathogenesis is conditioned by the absorption of the bacterium on the host, a process that is mediated by the components of its external membrane, both the proteins and the lipopolysaccharides (LPS).[3] In the latter case, the interaction is based on the recognition of a portion of the lipopolysaccharide, the so-called epitope, by receptor proteins situated on the plant cell wall. Actually, it is possible to saturate these receptors with an LPS solution or with a non-pathogenic bacterium having similar adsorption properties, leading to the protection of the plant from the bacterial action.

Within this framework, *A. radiobacter* acquires its importance because it competes with the pathogenic *A. tumefaciens* during the recognition process and, in addition, some strains produce a special bacteriocin, named agrocin 84, that is able to block DNA synthesis on nopaline-dependent *Agrobacteria*.[4]

Despite the wealth of information regarding the biological role of the LPS components from the *Agrobacterium* genus, to date, only few data are available regarding their chemical structures.[5,6] Since such information may play a key role in the comprehension of the adhesion mechanism of the bacterium on the plant cell wall, we investigated the chemical structure of the LPS from *A. radiobacter*.

Results and Discussion

Agrobacterium radiobacter, strain DSM 30199 (representative of group M2−1), possesses an S-type LPS as shown

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by the typical ladder appearance in the upper part of the gel electrophoresis (not shown).

The aqueous phase of the phenol/water treatment was purified by size-exclusion chromatography (SEC) to remove other contaminants, such as low-molecular-weight glucan and nucleic acid-based materials.

The purified fraction was subjected to compositional analysis, which revealed the presence of 3-hydroxymyristic acid together with minor amounts of palmitic, 3-hydroxypalmitic, 2-hydroxypalmitic and stearic acids.

The monosaccharide composition was obtained by GC-MS of acetylated *O*-methyl glycoside derivatives (Figure 1), which showed traces of 4-acetamido-4,6-dideoxyhexose, mannose, galactose, glucose and Kdo, as well as an intense peak at 24.7 min, attributed to a heptose residue on the basis of its retention time and of its characteristic ion at *m*/*z* 403.

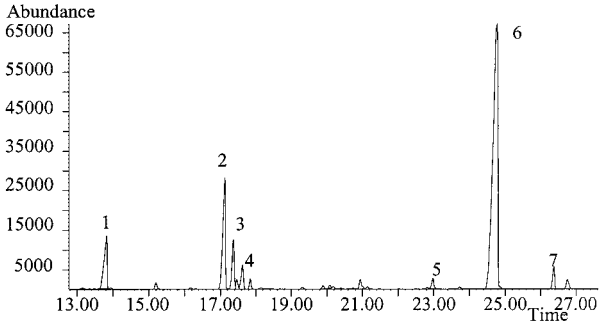

Figure 1. GC-MS chromatogram of the purified LPS fraction; the following peaks are recognised on the bases of their EIMS fragmentations and by comparison with opportune standards: (1) 4-acetamido-4,6-dideoxyhexose; [2, mannose; (3) galactose; (4) glucose; (5) 2-acetamido-2-deoxyglucose; (6) L-*glycero*-D-*manno*-heptose; (7) Kdo]

The relative stereochemistry of this heptose unit was determined by GC-MS by comparison with authentic standards: its retention time was coincident with that of L-*glycero*-D-*manno*-heptose, but, since this analysis could not provide the absolute stereochemistry, the possibility of having the enantiomer residue, D*-*glycero*-L*-*manno*-heptose, was not ruled out. Methylation analysis showed an intense peak at 20.5 min that originated from the partially methylated alditol acetate derivative of the heptose residue; its EIMS spectrum (Figure 2) showed the diagnostic ions at *m*/*z* 89, 118, 205, 234 and 350 that suggested that the heptose residue was present as a pyranose ring and substituted at the O-3 position. Some other minor signals were recognised by their fragmentation patterns, such as terminal 4-acetamido-4,6-dideoxyhexose, terminal galactose, terminal heptose (a small signal covered by an impurity), 2-substituted glucose and galactose, 3-substituted mannose, 3,6-substituted mannose, 3-substituted 4-acetamido-4,6-dideoxyhexose, and 3,7-disubstituted heptose.

The LPS was hydrolysed typically using 1% AcOH to obtain the O-chain polysaccharide part and to remove the lipid A moiety. New information was obtained by analysis of

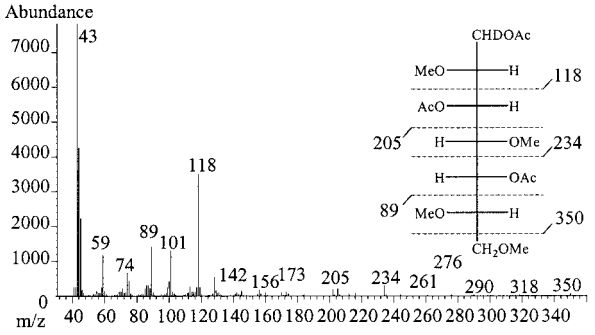

Figure 2. EI Mass spectrum of the partially methylated alditol acetate of the heptose residue; on the right-hand side, the corresponding structure is represented together with the more-diagnostic fragmentations

the ^{13}C NMR spectrum (Figure 3, a, Table 1) of the purified O-chain fraction; actually, it contained seven major signals, which suggested that the O-chain polysaccharide from the LPS possessed a homopolymeric structure with a single heptose residue as its repeating unit. In particular, the C-1 and C-5 signals at δ = 102.2 and 72.2 ppm, respectively, proved that the heptose was α-configured and the relatively low-field displacement of the C-3 carbon atom (δ = 77.7 ppm), when compared to a non-substituted heptose (Table 1, unit X[7]), suggested that the residue is substituted at O-3, in agreement with the methylation data. The ^{1}H NMR spectrum (Figure 3, b) was not in contrast with the above assumptions, but it was less informative because of overlapping of many signals.

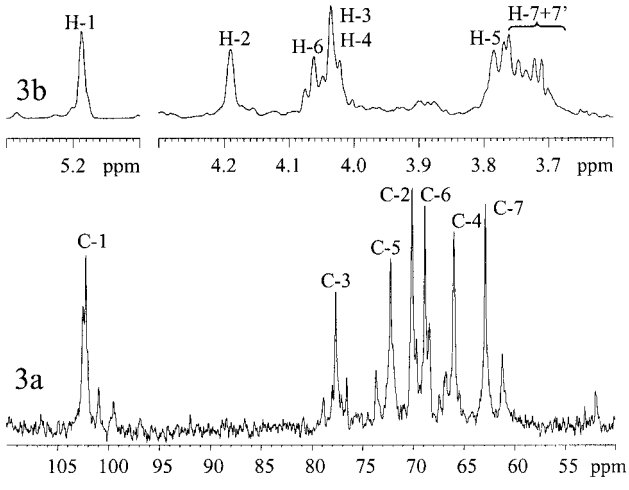

Figure 3. a) 75 MHz ^{13}C NMR spectrum and b) 500 MHz 1H NMR spectrum of the O-chain fraction from *A. radiobacter* DSM 30199. Assignments were performed on the basis of corresponding DQ-COSY and gsHSQC spectra.

The complete assignment of the resonances in the ^{1}H and ^{13}C NMR spectra (Table 1) was performed by combining the data with information from the DQ-COSY (Figure 4) and the gsHSQC spectra.

Table 1. ^{1}H (500 MHz, printed in bold), and ^{13}C (75 MHz) NMR spectroscopic chemical shifts and coupling constants [$^3J_{H,H}$ (printed in italics)] of the O-chain polysaccharide from *A. radiobacter* DSM 30199, and the ^{13}C NMR spectroscopic chemical shifts of the Smith-degraded product and the terminal heptose taken as a reference

	1	2	3	4	5	6	7-7′
3)-α-Hep-(1→	**5.19**	**4.19**	**4.04**	**4.04**	**3.78**	**4.06**	**3.77−3.72**
	–	–	–	–	$^3J_{4,5}$ = *7.3*	$^3J_{6,7'}$= $^3J_{6,7}$ = *6.9*	–
	102.2	70.1	77.7	66.0	72.2	68.8	62.9
3)-α-Man-(1→	102.4	69.9	78.4	66.3	73.8	61.3	–
t-α-Hep[a]	102.3	70.2	70.7	66.5	72.7	69.7	63.5

[a] Unit X from E. Vinogradov and K. Bock.[7]

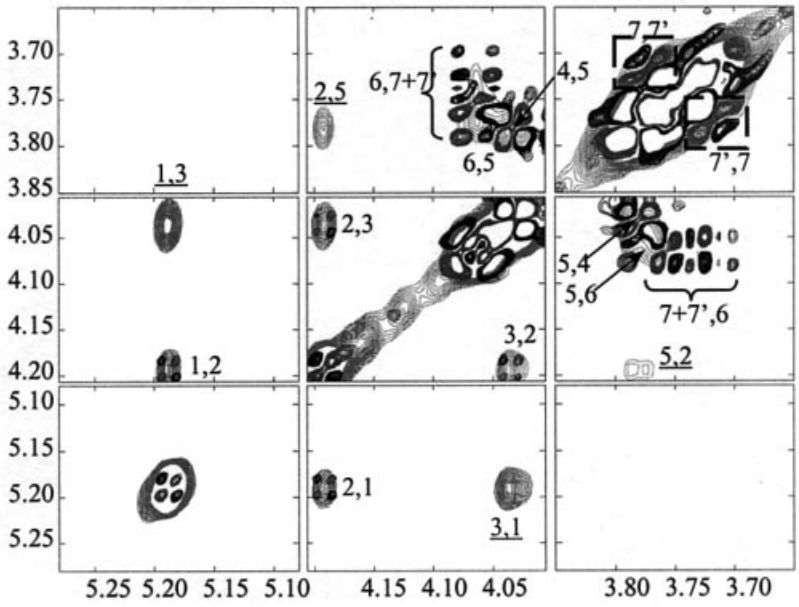

Figure 4. 500 MHz DQ-COSY (black and grey lines) and NOESY (light-grey lines) spectra of the O-chain from *A. radiobacter* DSM 30199; NOESY assignments, differently from those of the COSY spectrum, are underlined

The NOESY spectrum (Figure 4) showed an intense correlation with a proton signal at δ = 4.04 ppm; the assignment of this cross peak to H-3 was based on the methylation data, because, in the spectrum, H-3 overlaped with H-4 signal.

The MALDI-TOF mass spectrum of the main fraction obtained by SEC of the O-chain is presented in Figure 5 (a). The low resolution of the spectrum was due, most likely, to the extreme polarity of the polymeric chain, which led to an extensive amount of fragmentation of the polysaccharidic chains; the spectrum, however, clearly depicted a Gaussian molecular mass distribution centred at ca. 5300 Da, which indicated the average MW of the native polysaccharide. To obtain a more-informative mass spectrum and to minimise the fragmentation of the polymer,[8] the same fraction was subjected to permethylation. The MALDI-TOF mass spectrum of the permethylated polysaccharide (see b in Figure 5) showed a series of peaks separated by 248 Da, as expected for permethylated heptoses. The heterogeneity inside each group of peaks was due to variations in the core oligosaccharide linked to the O-chain moiety and to the typical under-methylation of the polysaccharide chain; these two factors normally led to a broad series of molecular peaks.

The absolute configuration of the heptose residue was determined studying the Smith-degraded product. This degradation protocol was selected because of its high selectivity: sodium metaperiodate cleaves oxidatively only the carbon−carbon linkages of *vicinal* hydroxy groups. Thus, in the present case, the only possible cleavage was that occurring between carbon atoms C-6 and C-7 of the heptose residue. In fact, we obtained a new homopolymer comprising α-(1→3)-mannose units (^{13}C NMR spectroscopic data in Table 1), as expected, and the absolute D configuration of these mannose residues was determined through the 2-*O*-octylglycoside derivative. Consequently, the heptose residue of the precursor polysaccharide possesses L-*glycero*-D-*manno* stereochemistry.

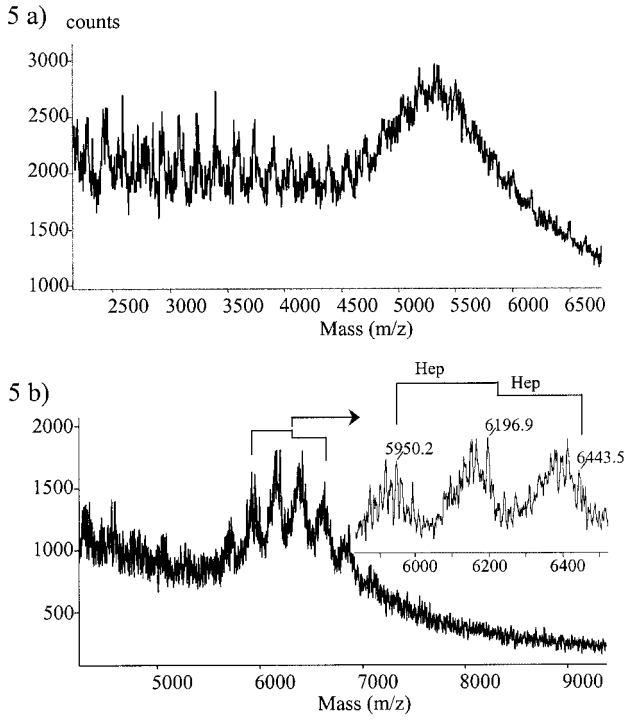

Figure 5. MALDI-TOF mass spectra of a representative fraction of the O-chain polysaccharide obtained by size-exclusion chromatographic (SEC): a) native sample analysed in the negative-ion linear mode; b) permethylated sample in the positive-ion linear mode. The expansion of the latter spectrum shows peak differences that indicate permethylated heptose units.

Conclusion

Spectroscopic information that is in agreement with the chemical data have allowed us to define the primary structure of the O-chain polysaccharide obtained from *A. radiobacter* strain DSM 30199 to be a homopolymer of L-*glycero*-D-*manno*-heptose units having the following formula: 3)-α-L-*glycero*-D-*manno*-heptose-(1→

This structure is the first reported for *Agrobacterium radiobacter* and, despite its apparent simplicity, it shows some peculiarities when compared with those obtained from other bacteria.

The L-*glycero*-D-*manno*-heptose is a very frequent residue found in all Gram-negative bacterial LPS reported so far, but its location is limited exclusively to the core region of the LPS molecule. To date, only one report describes its occurrence in the O-chain moiety: the LPS from *Pseudomonas cepacia* IMV 673/2.[9] In this case, the O-chain comprises a trisaccharide repeating unit in which one residue is a 2-substituted α-L-*glycero*-D-*manno*-heptose.

In all other LPSs, the heptose units present in the O-chain moiety always possesses a different absolute configuration. The O-chain from the LPS of *Helicobacter pylori* strain D4 is a heptan polysaccharide having a trisaccharide repeating unit,[10] in which the only monosaccharide residue is a D-*glycero*-D-*manno*-heptose unit, as is also found in the complex O-chain polysaccharide from the bacterium *Vibrio cholerae* O:3.[11] Other examples are represented by two bacterial LPSs from *Yersinia pseudotuberculosis* serovar 1A[12] and *Eubacterium saburreum*, strain T27,[13] whose antigenic moieties are constituted by 6-deoxy-D-*manno*-heptose and D-*glycero*-D-*galacto*-heptose units, respectively.

A careful examination of the occurrence of L-*glycero*-D-*manno*-heptose points to two relevant features: it is more frequent than the other isomers in the core region of the LPS, but it seldom occurs in the O-chain moiety. This evidence may be important to comprehend the molecular mechanism involved in the plant−host recognition. *Agrobacterium radiobacter* offers, to the plant cell wall receptors, one of the most widespread and characteristic residues, L-*glycero*-D-*manno*-heptose, that is the constituent of the majority of the core LPS. It is noteworthy, in this case, that this monosaccharide is highly exposed on the surface of the membrane, which is in contrast with the situation that exists when it is located in the core region, where it is partially hidden from the O-chain.

The bacterium may profit from the accessibility of this residue and set privileged interactions with the plant receptors for this epitope, feeding itself with the nutrient leaking from the plant, and preserving the host from the action of other potentially phytopathogenic bacteria.

Experimental Section

***Agrobacterium radiobacter* and Bacterial Cultivation:** The *A. radiobacter* strain DSM 30199[14] (representative strain of group M2−1, cluster 1) was grown at 27 °C in liquid shake culture (200 rpm) in Nutrient Broth (DIFCO) for 18 h (early stationary phase).
The bacterial suspension was centrifuged (3500 g for 5 min) and the harvested cells were washed sequentially with 0.85% NaCl, ethanol, acetone and diethyl ether. Typically, 10 L of culture yielded 1.4 g of dry cells.

Isolation and Purification of the LPS Fraction: Dried cells were extracted according to the phenol/water method.[15] Both phases were separately dialyzed against distilled water, freeze-dried and screened by 12% SDS-PAGE[16] on a miniprotean gel system from Bio-Rad; the samples (4 μg) where run at a constant voltage (150 V) and stained according to the procedure of Kittelberger and Hilbink.[17] Lipopolysaccharide material was found exclusively in the water phase.
The LPS fraction (100 mg) was further purified from nucleic material, and low-molecular-weight glucane, on a Sephacryl HR 400 (Pharmacia, 1.5 × 90 cm, eluent NH_4HCO_3 50 mM, flow 0.4 mL/min); the eluate was monitored using an R.I. refractometer (K-2310 Knauer) and the collected peaks were screened again by SDS-PAGE to give the LPS fraction [48 mg (6.0% yield respect dry cells)].

Analysis of the Chemical Composition: Monosaccharides were analysed as acetylated *O*-methyl glycoside derivatives and fatty acids as methyl esters, as reported.[6]
Absolute configuration was determined using the periodate-degraded O-chain fraction; the mannose configuration was deduced by analysis of the chiral 2-octyl derivatives according to the procedure of Leontein et al.[18]
Glycosyl-linkage analysis of LPS was performed according to the procedure of Sandford and Conrad;[19] the permethylated lipopolysaccharide was recovered in the organic layer of the water/chloro-

form extraction and converted into its partially methylated alditol acetates.[20]

GC-MS analysis conditions for all the derivatives mentioned above were the same; spectra were acquired on an Agilent 5973 instrument, using an SPB-5 capillary column (Supelco, 30 m × 0.25 i.d.; flow rate, 0.8 mL/min; He carrier gas) and the following temperature program: 150 °C for 5 min, 150 → 300 °C at 5.0 °C/min, 300 °C for 15 min. The mass spectra were recorded using an ionisation energy of 70 eV and an ionising current of 0.2 mA.

Isolation of the O-Specific Polysaccharide Fraction: Purified LPS fraction (50 mg) was dissolved in 1% acetic acid solution (2 mL), and kept at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000 rpm for 20 min and the clear supernatant was freeze-dried. The O-chain was purified by GFC on a Sephacryl HR 300 (Pharmacia, 1.5 × 70 cm, 50 mM NH_4HCO_3; flow rate: 0.4 mL/min); the eluate was monitored by its refractive index as above mentioned. The O-chain was isolated in ca. 60% yield from the LPS.

Smith Degradation Product of the O-Chain: The periodate-degraded product was obtained from the O-chain according to a procedure reported in literature.[21]

Acquisition of NMR Spectra: 2D NMR spectroscopy experiments were performed using a Varian Inova 500 [Consortium INCA (L488/92, Cluster 11)] equipped with a reverse probe operating at 25 °C. Chemical shifts of the spectra recorded in D_2O are expressed on the δ scale relative to internal acetone (δ = 2.225 and 31.4 ppm). Two-dimensional spectra (DQ-COSY, NOESY and phase-sensitive gradient-HSQC) were measured using standard Varian software.

For the homonuclear experiment, 256 FIDs of 1024 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 1024 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, processing and plotting was performed using the standard Bruker Xwin NMR 1.3 program.

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

MALDI-MS Analysis: MALDI-TOF analyses were conducted using an Applied Biosystems (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated through 24 kV. Insulin and myoglobin were used for external calibration. To better determine the MW distribution of the O-chain polysaccharide,[22] a sample (370 μg) was size-fractionated on a gel permeation chromatographic (GFC) system consisting of a Waters 515 HPLC pump, a TosoHaas TSKgel G3000 PWXL column (7.8 mm × 30 cm, Tosoh Biosep GmbH) and a Waters 410 differential refractometer as detector. Microfractions, obtained using H_2O as eluent at a flow rate of 0.8 mL/min, were collected every 12 s and subsequently dried on a centrifugal concentrator (SpeedVac Thermo Savant, USA). Selected fractions of the chromatographic peak were finally diluted in 0.1% CF_3CO_2H (20 μL) and mixed together (1:10) with the matrix solution {recrystallized 2,5-dihydroxybenzoic acid [30 g/L in 0.1% CF_3CO_2H/acetonitrile (80:20)]} before running the spectra using negative polarity.

Permethylation of the same fractions was performed in the presence of NaOH as described previously.[23] In this case, MALDI analyses were performed under positive polarity using 2,5-dihydroxybenzoic acid (30 g/L in methanol) as the matrix solution.

Acknowledgments

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