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Note

Structure of the O-specific polysaccharide of *Mesorhizobium huakuii* IFO15243T

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Abstract

The structure of the O-specific polysaccharide isolated by mild acid hydrolysis of the lipopolysaccharide of *Mesorhizobium huakuii* IFO15243T was studied using methylation analysis and various one- and two-dimensional ^{1}H and ^{13}C NMR experiments. The O-antigen polysaccharide was found to be linear polymer constituted by a trisaccharide repeating unit of the following structure:

→2)-α-L-6dTal*p*-(1→3)-α-L-6dTal*p*-(1→2)-α-L-Rha*p*-(1→

Keywords: O-Specific polysaccharide; Lipopolysaccharide; *Mesorhizobium huakuii*

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1. Introduction

Mesorhizobium huakuii is a member of the *Rhizobiaceae* family [1,22]. The cell envelope of Gram-negative bacteria, such as mesorhizobia, is built up of three layers: the cytoplasmic membrane, the peptidoglycan layer and the outer membrane. In the outer leaflet of the latter the lipopolysaccharide molecules are anchored by their lipid A moiety. The interaction between divalent cations (Ca^{2+}, Mg^{2+}) and negatively charged phosphate and uronic acid residues as well as the keto-deoxyoctonate acid (Kdo) residues of the inner core and lipid A region stabilise this almost impermeable surface structure. The O-chain, the distal part of LPS, is in direct contact with the environment. A lot of evidence support the proposition of the involvement of lipopolysaccharide in the nodule development process [2,6]. The O-antigens of rhizobia show a high variability from genus to genus and even from strain to strain within one genus [2]. The O-chains are often comprised of deoxysugars, methylated deoxysugars, uronic acids as well as heptosyl residues and some of them may be highly acetylated. In certain rhizobial strains there are polymers of repeating units [2,4–6,18,20] whereas in other strains they are homopolymers [2,3,19]. The highly hydrophobic O-chain isolated from *Mesorhizobium loti* NZP2213 LPS was described as a homopoly-

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mer built up of α-(1 → 3)-linked 2-*O*-acetyl-L-6-deoxytalose [3]. The present work deals with the structure of the O-specific polysaccharide of *Mesorhizobium huakuii* IFO15243T lipopolysaccharide.

2. Results and discussion

The lipopolysaccharide was isolated from bacterial cells by extraction with phenol–water and was purified by ultracentrifugation [7]. The LPS was cleaved into lipid A and polysaccharides (PS) with 1% acetic acid at 100 °C for 3 h. Water soluble polysaccharides were subjected to gel filtration chromatography on Sephadex G50. The O-chain PS was eluted from the column in the void volume. Complete acid hydrolysis of O-specific PS revealed the presence of 6-deoxytalose and rhamnose identified by GLC–MS as the alditol acetates. The molar ratios between these sugars were close to 2:1, respectively. The GLC analysis of trimethylsililated (−)-2-butyl glycoside derivatives showed the L configuration for both monosugars as evidenced using authentic sugar standards. Due to methylation analysis of O-antigen the 2- and 3-linked 6-deoxytalose and 2-linked rhamnose in the following molar ratios 1.10: 1.00: 0.95 were obtained.

The ^{13}C NMR spectrum of the O-specific polysaccharide (Fig. 1) showed a trisaccharide repeating unit. The spectrum contained three signals in the anomeric region in the range 98.07–102.21 ppm, 12 signals of sugar ring carbon atoms bearing oxygen in the region from 66.74 to 78.93 ppm and two signals for methyl groups of 6-deoxyhexoses at 17.60 and 16.24 ppm. The last one had almost twice the intensity as that at 17.60 ppm. The ^{1}H NMR spectrum of the O-chain polysaccharide contained, inter alia, signals for three anomeric protons and in the high-field region the multiplet derived from overlaping of three dublets of rhamnose and two 6-deoxytalose methyl protons (Fig. 2). Therefore the polysaccharide has a trisaccharide repeating unit composed of two residues of 6-L-deoxytalose and one residue of L-rhamnose. Only two small signals representing *O*-acetyl groups were detected. The comparison of the integrated areas of anomeric protons with areas of *O*-acetyl signals showed that less than one per 30 sugar residues were bearing acetyl groups.

The ^{1}H NMR spectrum of the O-specific polysaccharide was completely assigned (Table 1) by using 2D shift-correlation spectroscopy, i.e., DQF COSY and TOCSY. Carbon resonances observed in ^{13}C NMR spectrum were assigned on the bases of HSQC experiment displaying $^{1}J_{\text{C–H}}$ correlations

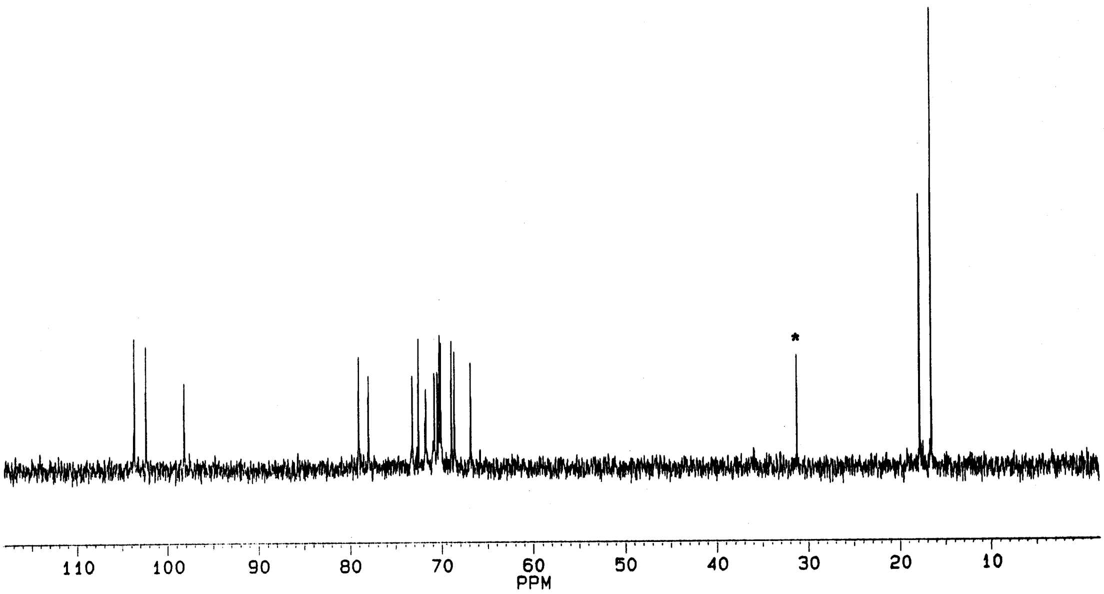

Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide. The signal marked with asterisk is the internal standard acetone δ_C 31.45 ppm.

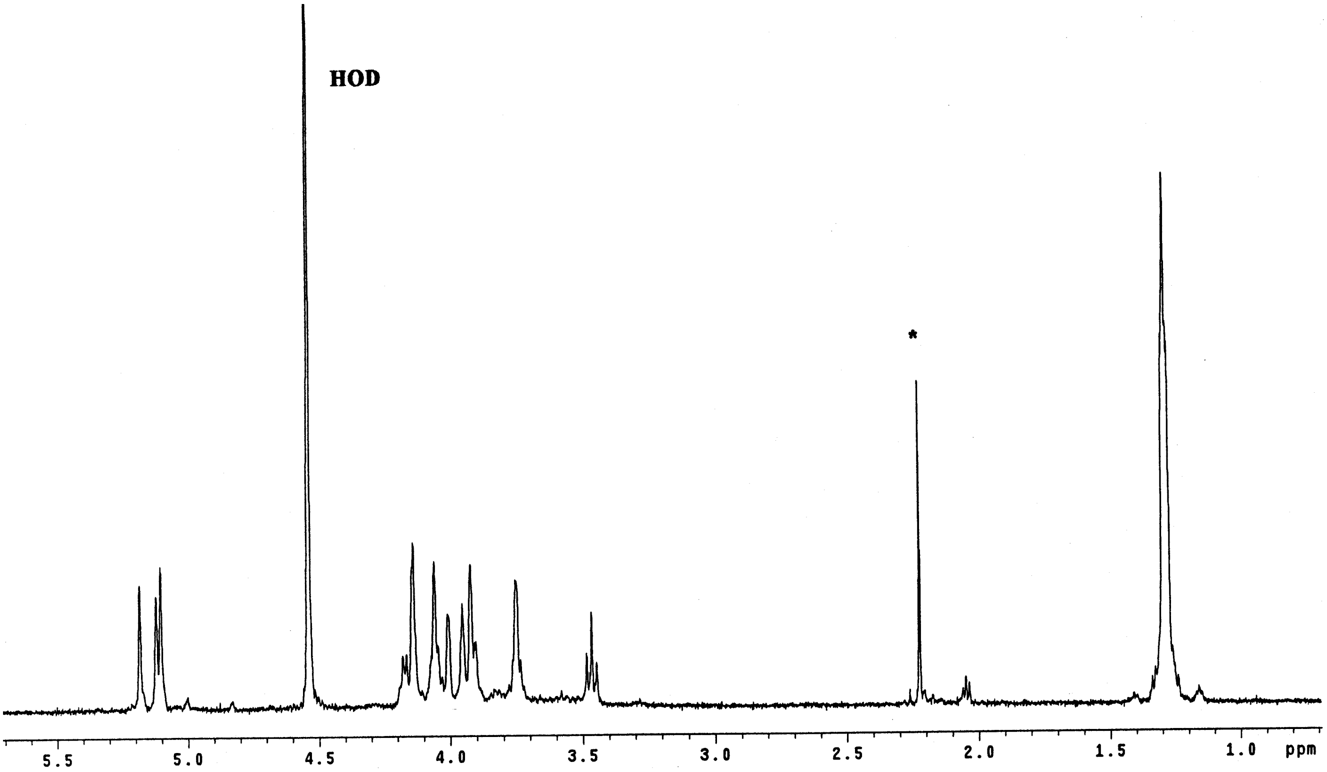

Fig. 2. ^{1}H NMR spectrum of the O-specific polysaccharide. The signal marked with asterisk is the internal standard acetone δ_H 2.225 ppm.

Table 1
^{1}H NMR data for the O-specific polysaccharide[a]

Data	H-1 $^3J_{1,2}$	H-2 $^3J_{2,3}$	H-3 $^3J_{3,4}$	H-4 $^3J_{4,5}$	H-5 $^3J_{5,6}$	H-6
→2)-α-L-6dTal*p*-(1→ (**T**)						
δ (ppm)	5.182	4.008	4.141	3.751	4.171	1.282
J (Hz)	s	~3	~3	bs	6.6	
→3)-α-L-6dTal*p*-(1→ (**T**′)						
δ (ppm)	5.103	4.059	3.955	3.923	4.052	1.279
J (Hz)	s	bs	~3	bs	6.6	
→2)-α-L-Rha*p*-(1→ (**R**)						
δ (ppm)	5.120	4.143	3.919	3.467	3.754	1.290
J (Hz)	s	bs	9.6	9.6	6.4	

[a] s, singlet; bs, broad singlet.

(Table 2). The rhamnose residue was identified by relatively large $^3J_{3,4}$ and $^3J_{4,5}$ values of ~10 Hz in comparison with the small values of ~3 Hz and less than 1 Hz (broad singlet), respectively, for 6-deoxytalose. ^{1}H NMR anomeric signals of all sugar residues were found to be broad singlets. Additionally, the $^1J_{C\text{-}1\text{–}H\text{-}1}$ coupling constant values measured in the gated decoupled ^{13}C NMR spectra demonstrated the α linkages of all the sugars (Table 2).

The 1D-NOE and ROE (or CAMELSPIN) experiments at pre-irradiation of the anomeric protons revealed the following interresidue correlations between transglycosidic protons: **T**H-1/**T**′H-3 at 5.182/3.955, **T**′H-1/**R**H-2 at 5.103/4.143 and **R**H-1/**T**H-2 at 5.120/4.008 ppm. Furthermore, the HMBC map (Fig. 3) showed cross-peak between: **T**H-1 and **T**′C-3, **T**′H-1 and **R**C-2 as well as between **R**H-1 and **T**C-2. All these facts indicated that 6-deoxy-

Table 2
^{13}C NMR data for the O-specific polysaccharide [a]

Data	C-1	C-2	C-3	C-4	C-5	C-6
→2)-α-L-6dTal*p*-(1→ (**T**)						
δ (ppm)	98.07	78.00	66.74	72.50	68.52	16.24
$^1J_{\text{C-1–H-1}}$ (Hz)	172.0					
→3)-α-L-6dTal*p*-(1→ (**T′**)						
δ (ppm)	103.57	70.21	71.73	70.04	68.77	16.24
$^1J_{\text{C-1–H-1}}$ (Hz)	173.6					
→2)-α-L-Rha*p*-(1→ (**R**)						
δ (ppm)	102.21	78.93	70.72	73.17	70.27	17.60
$^1J_{\text{C-1–H-1}}$ (Hz)	170.0					

[a] s, singlet; bs, broad singlet.

talose (**T**) was linked in a α-(1→3) linkage to 6-deoxytalose (**T′**), while this sugar was glycosidically bound to rhamnose (**R**) via an α-(1→2) linkage, which also was connected by an α-(1→2) linkage with the second 6-deoxytalose (**T**) in the following repeating unit. Moreover, the presence of intraresidue response of H-2 for pre-irradiation of anomeric protons and no NOE effect on H-3 and H-5 confirmed the α linkages among sugars.

In conclusion, the NMR experiments are in agreement with the analytical data and indicate the following structure for the trisaccharide repeating unit.

T **T'** **R**

→2)α-L-6dTal*p*(1→3)α-L-6dTal*p*(1→2)α-L-Rha*p*(1→

Rhizobial O-chains are often rich in deoxysugars [2–5]. In some cases they may be highly acetylated [3,4]. The pathway of 6-deoxytalose and rhamnose biosynthesis studied in *Actinobacillus actinomycetemcomitans* revealed as common precursor, the dTDP-6-deoxyhexosyl-4-ulose [8]. Probably, this or a similar pathway may be present and active in the *Mesorhizobium* cells.

Formerly, 6-deoxytalose had been described as a relatively rare constituent in natural products but is now more frequently found mostly as a component of bacterial polysaccharides [3,4,6,8–11]. The second component (rhamnose) of O-specific chain is a common constituent of microbial polysaccharides and particularly of bacterial LPS values [21]. This work shows, as a previous study [3] in *Mesorhizobium*, an O-specific chain built up exclusively of 6-deoxysugars. Our preliminary immunochemical studies revealed also the presence of deoxysugars, especially of 6-deoxytalose, in other *Mesorhizobium* lipopolysaccharides.

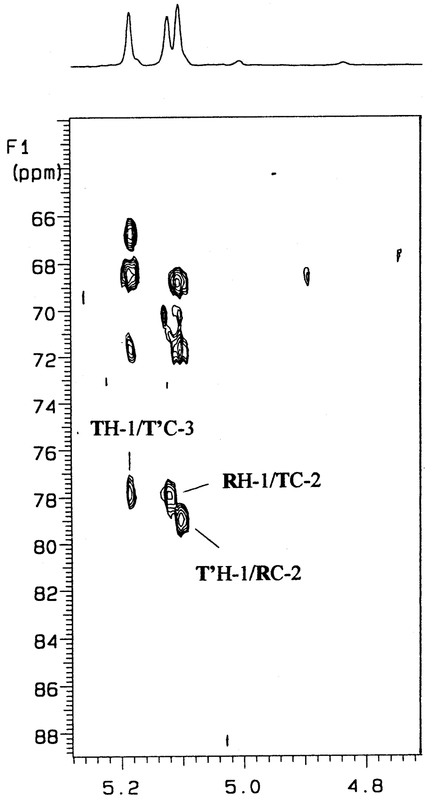

Fig. 3. Partial zone of the 2D HMBC spectrum of the O-specific polysaccharide. The map (F1, δ ^{13}C 64–88/F2, δ ^{1}H 5.30–4.80) shows connectivities involving anomeric ^{1}H resonances. **T**, **T′** and **R** represent the two 6-deoxytalose and the rhamnose residues respectively. Only transglycosidic correlations were marked.

3. Experimental

Bacterial strains, growth, and isolation of lipopolysaccharide and polysaccharides.—*Mesorhizobium huakuii* IFO15243T strain was obtained from the Institute for Fermentation Osaka, Japan. Bacteria were grown at 28 °C in liquid mannitol–yeast extract medium 79CA [12] and were aerated by vigorous shaking. Cells were centrifuged at 10,000*g*, washed twice with saline and once with distilled water. The bacterial mass was extracted by the hot phenol–water procedure [7]. Water layer was dialysed against tap and finally against distilled water. The crude LPS was purified by repeated ultracentrifugation at 105,000*g* for 4 h. Solution of LPS (5 mg mL^{-1}) in aq 1% AcOH was kept at 100 °C for 3 h. The lipid precipitate was removed by centrifugation and the polysaccharides were separated by gel permeation chromatography on Sephadex G50 fine column; (80 × 1.6 cm) with 1% AcOH as an eluent.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M TFA (100 °C, 4 h). The sugars were converted into alditols acetates [13]. The absolute configuration of the sugars were determined using (–)-2-butanol for glycoside preparation [14]. Methylation was done according to the method of Hakomori [15]. Methylated polysaccharides were purified on Sep-Pak C_{18} cartridge [16]. The resulting material was solvolysed in 90% formic acid (60 min, 80 °C) [17] and then hydrolysed in 2 M TFA (100 °C, 4 h) and reduced with $NaBD_4$. Partially methylated alditols were converted into acetate derivatives.

General methods.—GLC–MS was carried out on a Hewlett–Packard gas chromatograph (model HP5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP 5971). Helium was the carrier gas and the temperature program was: initially 150 °C for 5 min, then raised to 310 °C at a ramp rate of 3 °C min^{-1}, final time 20 min.

NMR spectroscopy.—^{1}H and ^{13}C NMR experiments were performed in D_2O solutions with acetone as an internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). 1D (NOE, ROE) and 2D (DQF COSY, TOCSY) ^{1}H NMR and ^{1}H/^{13}C ge-HMQC (gradient enhanced-HMQC) and ge-HMBC experiments were carried out on Varian Unity plus 500 instrument at 50 °C using standard Varian software. 1D ^{13}C NMR was obtained with a Bruker DRX-500 Avance spectrometer in D_2O at 60 °C.

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