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Note

Structural analysis of the O-polysaccharide of the lipopolysaccharide from *Azospirillum brasilense* Jm6B2 containing 3-*O*-methyl-D-rhamnose (D-acofriose)

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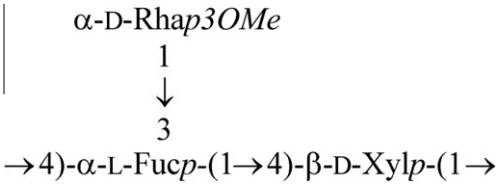
ABSTRACT

Two types of neutral O-polysaccharides were obtained by mild acid degradation of the lipopolysaccharide isolated by phenol–water extraction from the asymbiotic diazotrophic rhizobacterium *Azospirillum brasilense* Jm6B2. The following structure of the major O-polysaccharide was established by composition and methylation (ethylation) analyses, Smith degradation, and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy:



where a non-stoichiometric (~60%) 3-O-methylation of D-rhamnose is indicated by italics.

Colonization of plants by nitrogen-fixing bacteria, subsequently leading to an increase in plant growth activity and productivity, results from selective enrichment of the rhizosphere in adapted microorganisms.[1,2] The regularities of formation of plant–microbe associations have been best studied with the example of *Azospirillum* bacteria.[3] A key physiological factor of *Azospirillum* adaptation in the rhizosphere is the production of specific surface-located glycopolymers, among which an important role is played by lipopolysaccharides (LPSs). It is bacterial surface polysaccharides that are involved in the adhesion and adsorption of the microorganisms to the roots of plants and determine the success of the initial stages of formation of bacteria–root associations.[4,5] Furthermore, the O-specific polysaccharides (OPSs) of LPSs project into the environment and include antigenic determinants as part of their structure. On the basis of a comprehensive analysis of the chemical structure and serological properties of the O-antigens, we previously proposed three *Azospirillum* serogroups. The OPS repeating units of the bacteria assigned to serogroup I are represented by linear oligosaccharides composed of four or five D-Rha residues.[6] *Azospirillum* serogroup II strains are characterized by the presence of heteropolysaccharide OPSs and by a cross-reaction with antibodies developed to the LPS of *A. brasilense* type strain Sp7. Serogroup III is represented by bacteria in which the OPS repeating units are formed from three α-L-Rha residues in the main chain and a terminal β-D-Glc, which is possibly acetylated.[7]

A. brasilense strain Jm6B2 was isolated from the roots of maize in Ecuador.[8] No data on the LPS structure of *A. brasilense* Jm6B2 have been hitherto reported. By double radial immunodiffusion, preliminary serological studies demonstrated an absence of a cross-reaction of the LPS of azospirilla belonging to the three serogroups mentioned above. Considering the important role played by living conditions and plant exudates in the formation of bacterial glycopolymer composition (phenotype, glycoform), we speculated that

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this strain has an O-antigen structure that is unique to azospirilla, with the O-antigen being adapted to associating with C4 plants in a tropical zone.

Bacterial cells were extracted with aq 45% phenol, and the LPS was recovered from the aqueous phase. Degradation of the LPS under mild acid conditions afforded a lipid sediment and a water-soluble carbohydrate portion, which was fractionated by GPC on Sephadex G-50 to give a high-molecular-mass polysaccharide (PS). Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the PS revealed 3-*O*-methylrhamnose (Rha3OMe), rhamnose, fucose, and xylose in the 1:1.2:1.4:1 ratios. Determination of the absolute configurations of the monosaccharides by GLC of the acetylated glycosides with (*S*)-2-octanol indicated that Fuc is L and Xyl is D. The D configuration of Rha and Rha3OMe was determined by ^{13}C NMR spectroscopy (see below).

Methylation analysis of the PS by GLC–MS of the partially methylated alditol acetates resulted in identification of 2,3,4-tri-*O*-methylrhamnose, 2-*O*-methylfucose, and 2,3-di-*O*-methylxylose as major components. When ethyl iodide was used in place of methyl iodide, both 2,4-di-*O*-ethyl-3-*O*-methylrhamnose and 2,3,4-tri-*O*-ethylrhamnose were detected. Therefore, the major polysaccharide is branched with lateral rhamnose and 3-*O*-methylrhamnose residues and a 3,4-substituted fucose residue at the branching point. In addition, minor 6-deoxy-3,4-di-*O*-methylhexose, 6-deoxy-3-*O*-methylhexose, 2,3,4,6-tetra-*O*-methylhexose, and 2,3-di-*O*-methylhexose were detected in methylation analysis, which could derive from a minor polysaccharide(s) or a LPS core.

The 1D ^{13}C NMR (Fig. 1) and 2D ^{1}H, ^{13}C HSQC spectra of the PS showed major signals for four anomeric carbons at δ 98.1–105.6, two CH_3-C groups (C-6) of Fuc and Rha at δ 16.4 and 18.0, one $HOCH_2$-C group (C-5 of Xyl) at δ 63.7 and other sugar ring carbons at δ 67.0–81.4 as well as one CH_3-O group at δ 57.5. The absence of signals from the region of δ 83–88 that are characteristic of furanosides[9] indicated that all monosaccharide residues are in the pyranose form. The ^{1}H NMR spectrum of the PS contained, inter alia, major signals for anomeric protons at δ 4.46 and 5.02–5.06, two CH_3-C groups (H-6) of Rha and Fuc at δ 1.26 and 1.30 and one CH_3-O group at δ 3.43.

The major series in the ^{1}H and ^{13}C NMR spectra were assigned using ^{1}H, ^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and HMBC experiments (Tables 1 and 2). The sugar spin systems were identified by tracing connectivities from H-1 to H-5 of Xyl, H-1 to H-6 of Rha and Rha3OMe and H-1 to H-4 of Fuc in the COSY and TOCSY spectra combined with the characteristic coupling patterns. The remaining Fuc signals were assigned by a H-4, H-5 correlation in the ROESY spectrum and a H-6, H-5 correlation in the COSY spectrum. Rha3OMe was identified by a correlation between protons of the *O*-methyl group and C-3 of Rha at δ 3.43/80.8 in the HMBC spectrum. NMR signals of the minor polysaccharide(s) could not be assigned owing to multiple overlaps with major signals.

The ^{13}C NMR spectrum of the PS (Fig. 1, Table 2) revealed downfield displacements of the signals for C-3 of Rha3OMe, C-3 and C-4 of Fuc, and C-4 of Xyl, as compared with their positions in the corresponding non-substituted monosaccharides.[9,10] The monosaccharide sequence in the repeating unit was determined by the following correlations between anomeric protons and protons at the linkage carbons in the 2D ROESY spectrum: Xyl H-1,Fuc H-4; Fuc H-1,Xyl H-4, Rha H-1,Fuc H-3, and Rha3OMe H-1,Fuc H-3 at δ 4.46/4.01; 5.02/3.71; 5.02/3.99, and 5.06/3.99, respectively.

The D configuration of Rha and Rha3OMe was inferred by analysis of effects of glycosylation on the ^{13}C NMR chemical shift[11] in the α-Rha*p*-(1→3)-L-Fuc*p* and α-Rha*p*3OMe-(1→3)-L-Fuc*p* disaccharide fragments of the polysaccharide. Particularly, the effect of +7.7 ppm on C-1 of Rha and Rha3OMe, determined as a difference between the C-1 chemical shifts in the free[10] and linked α-Rha*p*, indicated different absolute configurations of the constituent monosaccharides, that is, the D configuration of Rha (in case of the same absolute configuration of Fuc and Rha, the effect on C-1 of Rha would not exceed 4 ppm).[11]

Based on these data, it was concluded that the major polysaccharide from the LPS of *A. brasilense* Jm6B2 consists of branched trisaccharide repeating units of two types, **1** and **2**, shown in Chart 1. As judged by the ratio of integral intensity of the ^{1}H NMR signals of Rha and Rha3OMe, the repeating units **1** and **2** are present in the ratio ~1.5:1.

Smith degradation[12] of the PS resulted in oxidation of the terminal Rha and 4-substituted Xyl residues. As a result, the α-Rha*p*3OMe-(1→3)-α-L-Fuc*p*-(1→2)-Gro glycoside **3** derived from the repeating unit **1** was isolated. Its structure was established by 2D ^{1}H and ^{13}C NMR spectroscopy as described above (the assigned ^{1}H and ^{13}C NMR chemical shifts are tabulated in Tables 1 and 2) and confirmed the structure of the major polysaccharide. No oligosaccharide products from the minor polysaccharide(s) were isolated by Smith-degradation, and its structure remains obscure.

A peculiar feature of the O-polysaccharide studied is the presence of 3-*O*-methyl-D-rhamnose (D-acofriose). O-Methylated monosaccharides are known to occupy the non-reducing terminus of a number of bacterial polysaccharides, mainly homopolysaccharides,[13] but occur rarely in repeating units, D-acofriose being one of the most common from them. Earlier, it has been reported as a constituent of

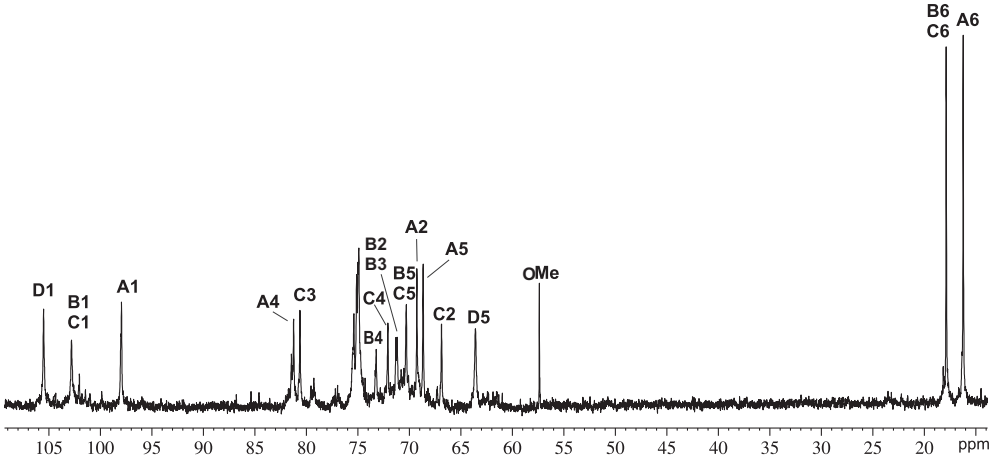


Figure 1. ^{13}C NMR spectrum of the polysaccharide from *A. brasilense* Jm6B2. Arabic numerals refer to carbons in sugar residues denoted as follows: A, Fuc; B, Rha; C, Rha3OMe; D, Xyl.

Table 1
^{1}H NMR chemical shifts (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	OMe
Major polysaccharide							
→3,4)-α-L-Fuc*p*-(1→ (**1**, **2**)	5.02	3.92	3.99	4.01	4.38	1.26	
→4)-β- D-Xyl*p*-(1→ (**1**, **2**)	4.46	3.41	3.57	3.71	3.30, 4.14		
α-D-Rha*p*3OMe-(1→ (**1**)	5.06	4.28	3.50	3.50	3.95	1.30	3.43
α-D-Rha*p*-(1→ (**2**)	5.02	4.03	3.80	3.48	3.95	1.30	
Glycoside ***3***							
α-D-Rha*p*3OMe-(1→	5.06	4.34	3.58	3.50	3.86	1.28	3.46
→3)-α-L-Fuc*p*-(1→	5.10	3.91	3.97	3.89	4.25	1.21	
Gro	3.72, 3.76	3.78	3.74				

Table 2
^{13}C NMR chemical shifts (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	OMe
Major polysaccharide							
→3,4)-α-L-Fuc*p*-(1→ (**1**, **2**)	98.1	69.4	75.2	81.4	68.8	16.4	
→4)-β-D-Xyl*p*-(1→ (**1**, **2**)	105.6	75.1	75.2	75.5	63.7		
α-D-Rha*p*3OMe-(1→ (**1**)	102.9	67.0	80.8	72.2	70.4	18.0	57.5
α-D-Rha*p*-(1→ (**2**)	102.9	71.3	71.3	73.4	70.4	18.0	
Glycoside ***3***							
α-D-Rha*p*3OMe-(1→	103.6	67.2	80.7	72.3	70.4	17.9	57.4
→3)-α-L-Fuc*p*-(1→	99.4	68.8	78.9	73.0	68.2	16.5	
→2)-Gro	61.7	80.1	62.8				

the O-units of *Pseudomonas syringae* pv. *phaseolicola*,[14] *Xanthomonas campestris* pv. *malvacearum*,[15] *Mesorhizobium amorphae*,[16] *Mesorhizobium loti*,[16] and *Burkholderia multivorans*.[17] In all bacteria studied except for *B. multivorans*, D-acofriose is present in a non-stoichiometric amount replacing partially D-rhamnose. 2-*O*-Methyl-L-rhamnose has been found in the repeating unit of one of the polysaccharides from the LPS of *A. brasilense* S17.[18]

1. Experimental

1.1. Bacterial strain, growth conditions, isolation and degradation of the lipopolysaccharide

A. brasilense Jm6B2 isolated from the roots of *Zea mays* was obtained from the microbial culture collection held at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov, Russia). The culture was continuously grown in a 10-L ANKUM-2 M fermentor at 30 °C in a liquid malate medium[19] to late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (15 g) were extracted with aq 45% phenol at 65–68 °C,[20] proteins and nucleic acids were precipitated by trichloroacetic acid and removed by centrifugation, and a LPS preparation was obtained by dialysis of the supernatant in a yield 2.1% of the dry cell mass.

A LPS sample (337 mg) was hydrolyzed with aq 2% HOAc at 100 °C for 6 h, a lipid precipitate was removed by centrifugation (13,000×g, 10 min), and the supernatant was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 Superfine in 0.05 M pyridinium acetate buffer pH 4.5 monitored with a Knauer differential refractometer. The yield of the high-molecular-mass PS was 30.3% of the LPS mass.

1.2. Chemical analyses

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h). Monosaccharides were analyzed by GLC as the alditol acetates[21] on an HP-5 capillary column using an Agilent 7820A GC system and a temperature gradient of 160 °C (1 min) to 290 °C at 7 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[22]

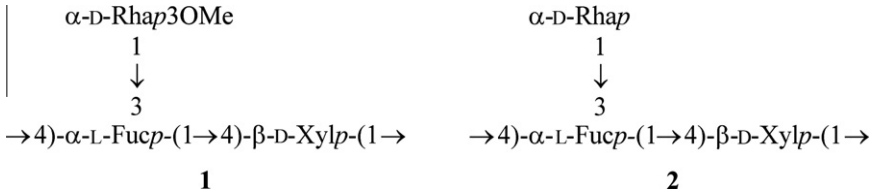
The PS was alkylated with CH_3I or C_2H_5I in dimethyl sulfoxide in the presence of sodium hydroxide. The products were hydrolyzed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially alkylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on an Agilent MSD 5975 instrument equipped with a HP-5 ms column using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.3. Smith degradation

A PS sample (75 mg) was oxidized with 0.1 M $NaIO_4$ (1.5 ml) in the dark at 20 °C for 48 h. After addition of an excess of ethylene glycol, reduction with $NaBH_4$ and desalting on a column (80 × 1.6 cm) of TSK HW-40 (S) in aq 1% AcOH, the products were hydrolyzed with aq 2% AcOH at 100 °C for 4 h and fractionated by GPC on TSK HW-40 (S) in aq 1% AcOH to yield glycoside **3** (8 mg).

1.4. NMR spectroscopy

A PS sample was deuterium-exchanged by freeze-drying from 99.9% D_2O. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance II 600 instrument at 30 °C. 3-(Trimethylsilyl)propanoate-d_4 (δ_H 0.0 ppm) and acetone (δ_C 31.45 ppm) were used as internal standards for calibration. 2D NMR experiments were performed using standard Bruker software. A mixing time of 200 ms was used in TOCSY and ROESY experiments. The HMBC spectrum was measured with a 60-ms delay for evolution of long-range couplings.



1 **2**

Chart 1. Structures of the repeating units **1** and **2** of the major polysaccharide from *A. brasilense* Jm6B2.

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