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Note

Structure of the O-polysaccharide of the lipopolysaccharide of *Azospirillum irakense* KBC1

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Abstract—The O-polysaccharide was isolated from the lipopolysaccharide of the plant-growth-promoting bacterium *Azospirillum irakense* KBC1 and studied by sugar and methylation analyses, Smith degradation and ^{1}H and ^{13}C NMR spectroscopy, including ^{1}H, ^{13}C HSQC and NOESY experiments for linkage and sequence analysis. The following structure of the branched hexasaccharide repeating unit of the O-polysaccharide with an unusually long side chain was established:

α-D-Gal*f*-(1→2)-α-L-Rha*p*-(1→3)-β-D-Man*p*-(1→3)-α-L-Rha*p*-(1→ 3)┐
→4)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→

Keywords: Lipopolysaccharide; O-antigen; Bacterial polysaccharide; Asymbiotic diazotroph; *Azospirillum irakense*

Azospirilla are Gram-negative plant-growth-promoting rhizobacteria, which are widely distributed in soils and establish associative relationships with roots of forage grasses, cereals and other nonlegumes. They fix atmospheric nitrogen and have a positive effect on plant growth and development by excreting phytohormones, vitamins and other biologically active substances into the rhizosphere.[1]

There is no serological classification of *Azospirillum* spp., and data on composition and structure of azospirilla lipopolysaccharide are scarce. Aiming at establishing a correlation between the structure of the lipopolysaccharide and its role in interaction of the bacteria with the plant roots, we have elucidated the chemical structure of the O-polysaccharide chain of the lipopolysaccharide of *Azospirillum brasilense* Sp245.[2] Now we report on a new structure of the O-polysaccharide of *Azospirillum irakense* KBC1.

Mild acid degradation of the lipopolysaccharide from *A. irakense* KBC1 resulted in a neutral high-molecular-mass O-polysaccharide, which was isolated by GPC on Sephadex G-50 and further purified from acidic contaminations by anion-exchange chromatography on DEAE-Trisacryl M. Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the polysaccharide showed the presence of rhamnose, mannose and galactose in the ratios ~3:1:2, respectively. Determination of the absolute configurations by GLC of the acetylated glycosides with a chiral alcohol indicated that rhamnose has the L-configuration, whereas mannose and galactose have the D-configuration.

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Methylation analysis of the polysaccharide, including GLC of the partially methylated alditol acetates, revealed derivatives from 2-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, 2,3,5,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose and 2,4,6-tri-*O*-methylmannose. Therefore, the polysaccharide is branched with a terminal galactose residue in the side chain and a 3,4-disubstituted rhamnose residue at the branching point. These data showed also that the terminal galactose residue is in the furanose form, whereas the other monosaccharides are in the pyranose form.

Each of the ^{1}H and ^{13}C NMR spectra of the polysaccharide contained signals for six monosaccharide residues and were assigned using ^{1}H, ^{1}H COSY, TOCSY, NOESY and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 1 and 2). The sugar spin systems were isolated by tracing connectivities in the TOCSY spectrum starting from H-1 for Gal*p* and Gal*f*, from H-1 and H-2 for Man*p* and from H-1 and H-6 for Rha*p*. The assignment within each spin system was performed using the COSY data. The identity of the monosaccharides was established by characteristic coupling pattern and $^{3}J_{H,H}$ constant values. The assignment for Gal*p* was completed by an H-1,H-5 correlation observed in the NOESY spectrum, which, together with a relatively large $J_{1,2}$ coupling constant of ~8 Hz, indicated the β-linkage of this residue. The α-linkage of Gal*f* was established by the C-1 chemical shift of δ 103.1 (the value for β-Gal*f* would be about δ 109), and the α-linkage of the three rhamnose residues (Rhap^{I}–Rhap^{III}) as well as the β-linkage of the mannose residue were determined by comparison of the C-5 chemical shifts with those of the corresponding α- and β-hexopyranosides[3] (Fig. 1).

The ^{13}C NMR chemical shift data (Table 2, Chart 1) revealed low-field displacements of the signals for the

Table 1. 500-MHz ^{1}H NMR data (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
O-Polysaccharide							
→4)-α-L-Rhap^{I}-(1→ 3 ↑	5.04	4.12	4.11	3.83	3.97	1.35	
→ 3)-β-D-Gal*p*-(1 →	4.58	3.61	3.71	4.02	3.65	3.76	3.80
→ 3)-α-L-Rhap^{II}-(1 →	5.10	4.29	4.03	3.61	3.88	1.31	
→ 3)-β-D-Man*p*-(1 →	4.92	4.31	3.72	3.72	3.42	3.81	3.94
→ 2)-α-L-Rhap^{III}-(1 →	5.05	4.03	3.89	3.45	3.96	1.28	
α-D-Gal*f*-(1 →	5.09	4.12	4.30	3.88	3.79	3.64	3.64
Smith-degraded polysaccharide							
→4)-α-L-Rhap^{I}-(1→ 3 ↑	5.03	4.12	4.12	3.82	3.95	1.36	
→ 3)-β-D-Gal*p*-(1 →	4.59	3.61	3.71	4.02	3.65	3.73	3.73
→ 3)-α-L-Rhap^{II}-(1 →	5.09	4.29	4.03	3.60	3.88	1.30	
β-D-Man*p*-(1 →	4.93	4.11	3.65	3.62	3.38	3.77	3.94

Table 2. 125-MHz ^{13}C NMRdata (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
O-polysaccharide						
→4)-α-L-Rhap^{I}-(1→ 3 ↑	103.3	71.2	79.9	78.6	69.3	18.5
→ 3)-β-D-Gal*p*-(1 →	104.2	71.6	82.2	69.8	76.0	62.1
→ 3)-α-L-Rha^{II} *p*-(1 →	103.3	71.3	80.1	72.5	70.4	17.8
→ 3)-β-D-Man*p*-(1 →	102.2	68.0	78.1	66.1	77.5	62.3
→ 2)-α-L-Rhap^{III}-(1 →	96.1	80.3	70.7	73.6	70.1	17.8
α-D-Gal*f*-(1 →	103.1	77.2	74.2	82.0	71.3	64.1
Smith-degraded polysaccharide						
→4)-α-L-Rhap^{I}-(1→ 3 ↑	102.9	71.1	79.9	79.0	69.5	18.6
→ 3)-β-D-Gal*p*-(1 →	103.7	71.5	82.4	69.8	76.1	62.3
→ 3)-α-L-Rhap^{II}-(1 →	102.9	71.5	79.9	72.6	70.3	17.9
β-D-Man*p*-(1 →	101.8	71.8	74.1	66.3	77.6	62.4

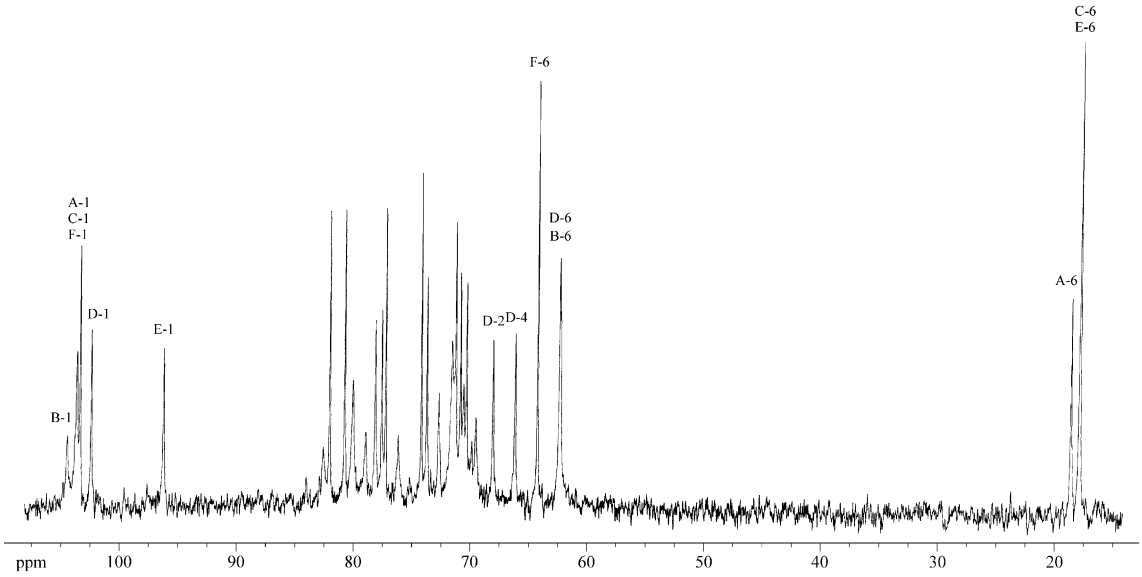

Figure 1. ^{1}C NMR spectrum of the O-polysaccharide of *A. irakense* KBC1, where A is α-L-Rha*p*^{I}, B—β-D-Gal*p*, C—α-L-Rha^{II}*p*, D—β-D-Man*p*, E—α-L-Rha*p*^{III} and F—α-D-Gal*f*.

Man*p*-(1→3)-α-L-Rha*p*^{II}-(1→3)┐
→4)-α-L-Rha*p*^{I}-(1→3)-β-D-Gal*p*-(1→

1

α-D-Gal*f*-(1→2)-α-L-Rha*p*^{III}-(1→3)-β-D-Man*p*-(1→3)-α-L-Rha*p*^{II}-(1→3)┐
→4)-α-L-Rha*p*^{I}-(1→3)-β-D-Gal*p*-(1→

2

Chart 1. Structures of the O-polysaccharide of *A. irakense* KBC1 (**1**) and Smith-degraded polysaccharide (**2**).

following carbons as compared with their positions in the corresponding nonsubstituted monosaccharides:[3] Man*p* C-3, Gal*p* C-4, Rha*p*^{I} C-3 and C-4, Rha*p*^{II} C-3 and Rha*p*^{III} C-2. These effects were due to glycosylation and defined the linkage pattern in the repeating unit. The C-2–C-6 chemical shifts of Gal*f* were close to the values for unsubstituted α-galactofuranose, which is in agreement with the terminal position of this monosaccharide shown by methylation analysis.

Determination of the sequence of the monosaccharides in the repeating unit using NOESY spectroscopy was ambiguous owing to overlapping of signals of several protons at the linkage carbon atoms. Therefore, the polysaccharide was subjected to Smith degradation, which resulted in oxidation of the terminal galactose and 2-substituted rhamnose residues in the side chain to give a modified polysaccharide. Assignment of its ^{1}H and ^{13}C NMR spectra was performed by shift-correlated experiments as described above for the polysaccharide (Tables 1 and 2). The following correlations between the anomeric protons and protons at the linkage carbon atoms in the ROESY spectrum of the modified polysaccharide: Rha*p*^{I} H-1,Gal*p* H-3 at δ 5.03/3.71; Gal*p* H-1,Rha*p*^{I} H-4 at δ 4.59/3.82; Rha*p*^{II} H-1,Rha*p*^{I} H-3 or H-2 at δ 5.09/4.12; Man*p* H-1,Rha*p*^{II} H-3 at δ 4.93/4.03. Choice in favour of the Rha*p*^{II} H-1,Rha*p*^{I} H-3 versus Rha*p*^{II} H-1,Rha*p*^{I} H-2 was made based on methylation and ^{13}C NMR chemical shifts data (see above), which showed that Rha^{I} is 3,4-disubstituted. These data defined the linkage pattern and the sequence of the sugar residues, and, hence, the Smith-degraded polysaccharide is branched and has the structure **1** shown in Chart 1.

Then, taking into account the data of the polysaccharide **1**, the correlations in the NOESY spectrum of the initial polysaccharide could be interpreted as follows: Rha*p*^{I} H-1,Gal*p* H-3 at δ 5.04/3.71; Gal*p* H-1,Rha*p*^{I} H-4 at δ 4.58/3.83; Rha*p*^{II} H-1,Rha*p*^{I} H-3 at δ 5.10/4.11; Man*p* H-1,Rha*p*^{II} H-3 at δ 4.92/4.03; Rha*p*^{III} H-1,Man*p* H-3 at δ 5.05/3.72; Gal*f* H-1,Rha*p*^{III} H-2 at δ 5.09/4.03. Based on these data, the O-polysaccharide of the lipopolysaccharide of *A. irakense* KBC1 is built up of branched hexasaccharide repeating units with an unusually long side chain and has the structure **2** shown in Chart 1.

1. Experimental

1.1. Growth of bacteria and isolation of the lipopolysaccharide and O-polysaccharide

A. irakense strain KBC1 isolated from rice roots[4] was kindly provided by the late Dr. Johanna Döbereiner (Embrapa Agrobiologia Rio de Janeiro, Brazil). The bacteria were continuously grown in a 10-L ANKUM-2M fermentor at 30 °C in a liquid malate medium[5] till the late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (10 g) were extracted with a phenol–water mixture,[6] and the lipopolysaccharide was purified by repeated ultracentrifugation (105,000*g*, 2×4 h). The yield of the lipopolysaccharide was 1.1% of the dry cell weight.

1.2. Isolation of the O-polysaccharide

The lipopolysaccharide (100 mg) was degraded with aq 1% AcOH for 4 h at 100 °C; the lipid precipitate was removed by centrifugation, and the water-soluble portion was fractionated by GPC on a column (56×2.6 cm) of Sephadex G-50 (S) using 0.05 M pyridinium acetate pH 4.5 as eluent and monitoring with a Knauer differential refractometer. The high-molecular-mass polysaccharide (31.5 mg) was further purified by anion-exchange chromatography on a column (20×1 cm) of DEAE-Trisacryl M in a stepwise gradient of 0.005, 0.01, 0.1, 0.25, 0.5 M sodium phosphate pH 6.3. The yield of the purified O-polysaccharide, which was eluted with the first buffer, was 15.5% of the lipopolysaccharide weight.

1.3. Sugar analysis

Hydrolysis of the polysaccharide was performed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were analysed by GLC as the alditol acetates[7] on an Ultra 2 capillary column using a Hewlett–Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[8]

1.4. Methylation analysis

Methylation of the polysaccharide was carried out with CH_3I in Me_2SO in the presence of methylsulfinylmethanide.[9] Hydrolysis of the methylated polysaccharide was performed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with an HP-5 column using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.5. Smith degradation

The polysaccharide (20 mg) was oxidised with 0.1 M $NaIO_4$ in the dark for 48 h at 20 °C, after adding an excess of ethylene glycol, reduction with $NaBH_4$ and desalting on a column (80×1.6 cm) of TSK HW-40 (S) in water, the product was hydrolysed with aq 2% AcOH for 2 h at 100 °C, reduced with $NaBH_4$, desalted by treatment with a KU-2 cation-exchange resin (H^+-form) and a Smith-degraded polysaccharide (8 mg) was isolated by GPC on TSK HW-40 (S).

1.6. NMR spectroscopy

Prior to measurements, samples were deuterium exchanged by freeze drying from D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 27 °C. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.45). A mixing time of 200 and 150 ms was used in TOCSY and NOESY experiments, respectively.

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