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Note

Structure of the O-specific polysaccharide of the lipopolysaccharide of *Azospirillum brasilense* Sp245

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Abstract

An O-specific polysaccharide was isolated from the lipopolysaccharide of a plant-growth-promoting bacterium *Azospirillum brasilense* Sp245 and studied by sugar analyses along with one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy, including NOESY. The polysaccharide was found to be a new rhamnan with a pentasaccharide repeating unit having the following structure:

→2)-β-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→

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Azospirilla are Gram-negative, asymbiotic diazotrophs belonging to the alpha subclass of proteobacteria. As plant-growth-promoting rhizobacteria, they are able to fix atmospheric nitrogen and have a positive effect on plant growth and development by excreting into the rhizosphere phytohormones, vitamins, and other biologically active substances.[1] Azospirilla do not associate with any particular plant species; they are widely present in soils and establish associative relationships with the roots of forage grasses, cereals, and other non-legumes.[2] The genus *Azospirillum* is divided into seven species, the most extensively studied species being *A. brasilense* and the first species of this genus described, *A. lipoferum*.[3]

Despite that the extensive literature that has been accumulated on the molecular mechanisms of the *Azospirillum*–cereals interaction, many details have yet to be elucidated. Glycopolymers, which are present on the bacterial surface, are considered to play an important role in the interaction. Extracellular and capsular polysaccharides of azaspirilla have been investigated,[4,5] whereas lipopolysaccharides (LPS) remain little studied. The O-specific polysaccharide structure has been studied in only one strain of *A. lipoferum*,[6] and no data on the core and lipid A moieties of the LPS are currently available. We are interested in isolation of surface glycopolymers of azospirilla and their studies for taxonomic purposes and for elucidation of their role in the interaction with plant roots. The particular interest in *A. brasilense* Sp245 studied in this work is associated with its ability to penetrate the root interior,[7,8] as distinct from most of the other strains of the genus, which colonize only the root surface.

The O-specific polysaccharide was isolated by mild-acid degradation of the LPS from *A. brasilense* Sp245 followed by GPC on Sephadex G-50. Sugar analysis of the polysaccharide, including determination of the absolute configurations, demonstrated the presence of D-rhamnose (D-Rha).

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for five anomeric carbons at δ 97.8–103.3, five methyl groups (Rha C-6) at δ 17.8–

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17.9, and twenty other sugar ring carbons in the region δ 68.4–79.0. Accordingly, the ^{1}H NMR spectrum of the polysaccharide (Fig. 2) contained signals for five anomeric protons at δ 4.82–5.20, five methyl groups (Rha H-6) at δ 1.30–1.33, and other sugar protons in the region δ 3.43–4.25.

These data showed that the polysaccharide is a D-rhamnan having a pentasaccharide repeating unit. The position of the C-6 signals near δ 18 and the absence from the ^{13}C NMR spectrum of signals in a lower field than δ 80 indicated that all rhamnose residues are in the pyranose form.[9]

The ^{1}H and ^{13}C NMR spectra of the polysaccharide (Tables 1 and 2) were assigned using 2D NMR experiments (COSY, TOCSY, and ^{1}H,^{13}C HSQC). Relatively high-field positions of the signals for H-1,3,5 of one of the rhamnose residues (Rha^{I}) at δ 4.82, 3.73, and 3.42, respectively, showed that this residue is β-linked, whereas the other four rhamnose residues (Rha^{II}–Rha^{V}) with the H-1,3,5 resonances in a lower field at δ 4.97–5.20, 3.85–4.04, and 3.75–4.21, respectively, are α-linked (compare published data for β- and α-rhamnopyranose[10]).

Low-field displacements to δ 78.1–79.5 of the signals for C-2 or C-3 of each rhamnose residue (Table 2), as compared with their positions in the non-substituted α-rhamnopyranose at δ 71–73,[9] showed that the polysaccharide is linear and revealed the positions of substitution of the monosaccharides. The glycosylation effects on the ^{13}C NMR chemical shifts of the rhamnose residues were in agreement with the absolute configuration of the monosaccharides and the configurations of the glycosidic linkages.[11]

A NOESY experiment revealed the following interresidue correlations between the anomeric and linkage protons: Rha^{I} H-1/Rha^{II} H-2 and H-3, Rha^{II} H-1/Rha^{III} H-3, Rha^{III} H-1/Rha^{IV} H-2, Rha^{IV} H-1/Rha^{V} H-2, and Rha^{V} H-1/Rha^{I} H-2 at δ 4.82/4.25 and 4.04, 5.09/3.85, 4.97/4.10, 5.12/4.12, and 5.20/4.07, respectively. Rha^{I} was characterized by intraresidue H-1,H-2,3,5 correlations, whereas the other four Rha residues showed only an H-1,H-2 correlation. These data confirmed the positions of substitution and the anomeric configurations of the monosaccharides and defined their sequence in the polysaccharide repeating unit.

Therefore, the O-specific polysaccharide of the LPS of *A. brasilense* Sp 245 has the following structure:

$$\rightarrow 2)\text{-}\beta\text{-D-Rha}p^{I}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p^{II}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-}$$

$$\text{Rha}p^{III}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{IV}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{V}\text{-}(1\rightarrow$$

To our knowledge this structure is new among natural polysaccharides. Remarkably, as in *A. brasilense*, in *Pseudomonas syringae*[12] and *Xanthomonas campestris*

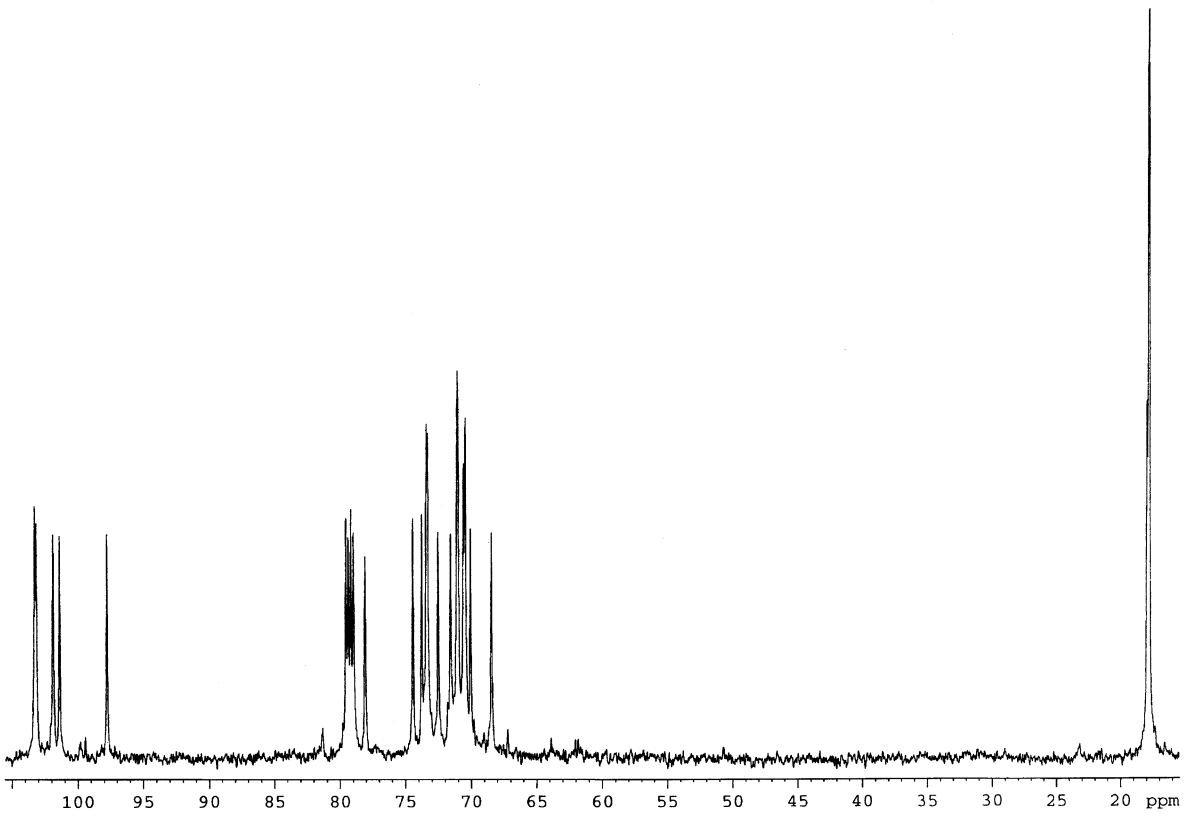

Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide of *A. brasilense* Sp245.

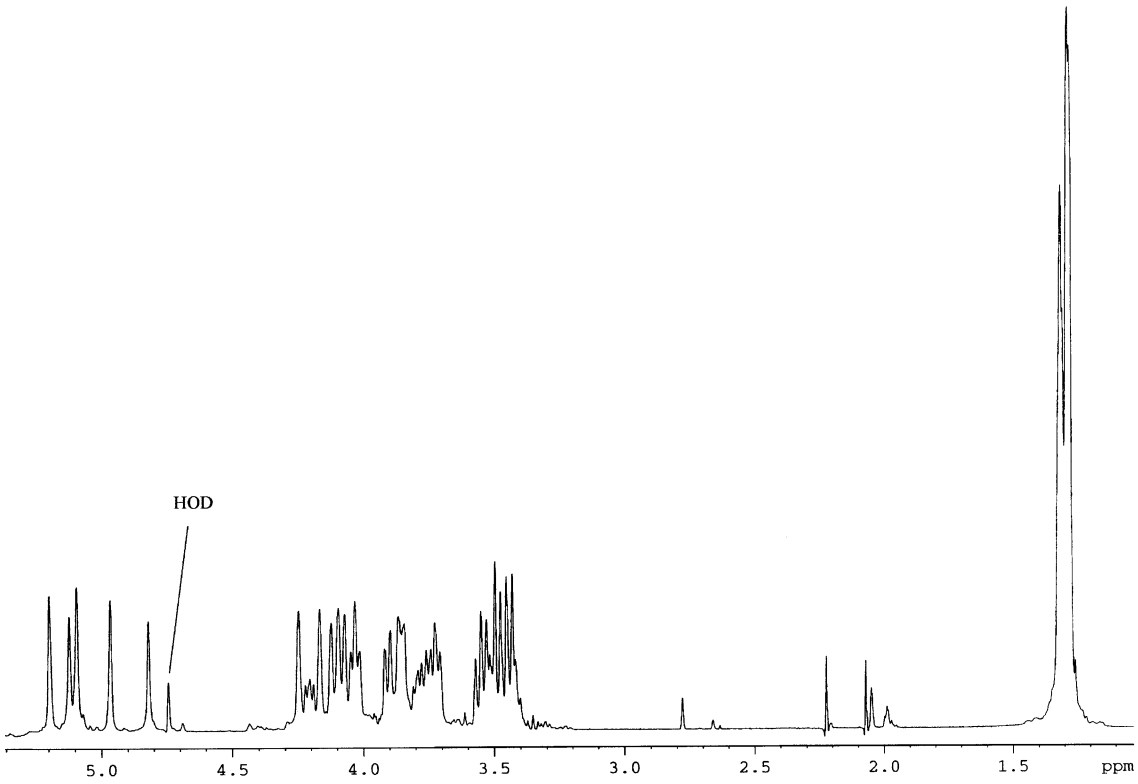

Fig. 2. 500-MHz ^{1}H NMR spectrum of the O-specific polysaccharide of *A. brasilense* Sp245.

Table 1
500-MHz ^{1}H NMR data of the O-specific polysaccharide (δ in ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
→2)-β-D-Rha*p*^{I}-(1→	4.82	4.07	3.73	3.43	3.42	1.33
→3)-α-D-Rha*p*^{II}-(1→	5.09	4.25	4.04	3.53	3.85	1.33
→3)-α-D-Rha*p*^{III}-(1→	4.97	4.17	3.85	3.55	3.79	1.30
→2)-α-D-Rha*p*^{IV}-(1→	5.12	4.10	3.91	3.49	3.75	1.30
→2)-α-D-Rha*p*^{V}-(1→	5.20	4.12	4.02	3.49	4.21	1.30

Table 2
125-MHz ^{13}C NMR data of the O-specific polysaccharide (δ in ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→2)-β-D-Rha*p*^{I}-(1→	97.8	79.0	74.4	73.3	73.8	17.9
→3)-α-D-Rha*p*^{II}-(1→	103.3	68.4	78.1	71.6	70.4	17.9
→3)-α-D-Rha*p*^{III}-(1→	103.2	71.0	79.2	72.5	70.6	17.8
→2)-α-D-Rha*p*^{IV}-(1→	101.9	79.4	71.0	73.4	70.0	17.8
→2)-α-D-Rha*p*^{V}-(1→	101.4	79.5	71.0	73.4	70.0	17.8

(Ref. 13 and references cited therein) pathovars, rhamnose (D or L) is the main or the only constituent of the O-specific polysaccharide and is thus rather common for phytopathogenic bacteria. This may be an indication that this sugar plays a role in the recognition and interaction between bacteria and plants.

1. Experimental

Growth of bacteria and isolation of the lipopolysaccharide and O-specific polysaccharide.—The culture of *A. brasilense* strain Sp245 isolated from surface-sterilized wheat roots[14] was obtained from Dr J. Döbereiner

(Embrapa Agrobiologia Rio de Janeiro, Brazil). The culture was continuously grown in a 10-L ANKUM-2 M fermenter at 30 °C in liquid malate medium[4] to late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (20 g) were extracted with phenol–water,[15] and the LPS was purified by GPC on a column (55 × 1.8 cm) of Sepharose CL-4B in 0.025 M NH_4HCO_3 (pH 8.3). The yield of the LPS was 2.25% of the dry cells weight.

The O-specific polysaccharide was obtained by degradation of the LPS with aq 1% HOAc for 4 h at 100 °C followed 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 (40 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 buffer (pH 6.3). The yield of the polysaccharide was 26.3% of the LPS weight.

Sugar analysis.—Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were analyzed by GLC as the alditol acetates 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. The absolute configurations were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[16]

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying from ^2H_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in ^2H_2O at 27 °C. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45). A mixing time of 200 and 150 ms was used in TOCSY and NOESY experiments, respectively.

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