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Note

Structural studies of the polysaccharides from the lipopolysaccharides of *Azospirillum brasilense* Sp246 and SpBr14

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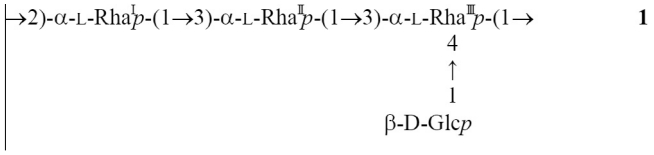
ABSTRACT

Lipopolysaccharides from closely related *Azospirillum brasilense* strains, Sp246 and SpBr14, were obtained by phenol–water extraction. Mild acid hydrolysis of the lipopolysaccharides followed by GPC on Sephadex G-50 resulted in polysaccharide mixtures. On the basis of sugar and methylation analyses, Smith degradation and ^{1}H and ^{13}C NMR spectroscopy data, it was concluded that both bacteria possess the same two distinct polysaccharides having structures **1** and **2**:



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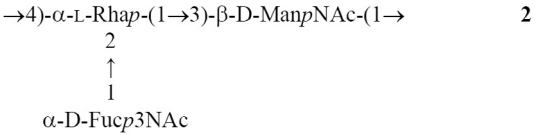

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Structure **1** has been reported earlier for a polysaccharide of *A. brasilense* 54 [Fedonenko et al., 2011][6] whereas to our knowledge structure **2** has not been hitherto found in bacterial polysaccharides.

Surface polysaccharides of soil bacteria are an important communication factor allowing microorganisms to build various forms of interactions in biocenosis. Mutualistic plant-bacterial associations increase the survival chances of both partners, and the success of their formation depends on the 'molecular dialogue' at the initial stages of the interaction. The Gram-negative N_2-fixing bacteria *Azospirillum* are well-known growth-promoting microorganisms that interact with a broad host range.[1,2]

Lipopolysaccharides (LPSs) are amphiphilic macromolecules that form the outer layer of the outer membrane of Gram-negative bacteria. In addition to structural and barrier functions, *Azospirillum* LPSs are involved in mechanisms of host recognition and adsorption and in induction of host responses.[3,4] The LPS molecule is composed of three moieties: lipid A, a hydrophobic domain that anchors the LPS molecule into the membrane and is responsible for biological activities of LPS; a core oligosaccharide and an O-specific polysaccharide (OPS), which protrudes into the environment and carries antigenic determinants. S-form LPS has all three moieties, whereas in R-form LPS, the carbohydrate portion is limited to the core oligosaccharide.

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Recently, structures of the OPSs of *Azospirillum* have been studied intensively. Several groups of the bacteria have been identified whose OPSs either have identical repeating units or share a common backbone structure.[5–7] Although azospirilla are not strictly specific towards their plant hosts, a selectivity in identifying a partner has been demonstrated for several strains,[8,9] which may be due to a convergent development of the bacteria residing in similar ecological niches. In this paper, we report structures of the OPSs isolated from the LPSs of two closely related *Azospirillum brasilense* strains, Sp246 and SpBr14.[10]

The LPSs were extracted from bacterial biomass by aqueous phenol and degraded under mild acidic conditions. Lipid A sediments were removed by centrifugation, the OPS-containing supernatants were separated from low-molecular fractions by GPC on Sephadex G-50. Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the OPSs from both strains revealed rhamnose, glucose, 2-acetamido-2-deoxymannose (ManNAc) and 3-acetamido-3-deoxyfucose (Fuc3NAc) in the ratio ~3.7:0.7:1:0.6. GLC of the acetylated (*S*)-2-octyl glycosides revealed the D configuration of Glc and the L configuration of Rha. The absolute configurations of ManN and Fuc3N were determined by ^{13}C NMR spectroscopy (see below).

GLC–MS analysis of the partially methylated alditol acetates derived from the methylated OPSs indicated the presence of 2,3,4,6-tetra-*O*-methyl-glucose, 2-*O*-methylrhamnose, 3-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidomannose (from ManNAc) and 3-deoxy-2,4-di-*O*-methyl-3-(*N*-methyl)acetamidofucose (from Fuc3NAc). Therefore, each OPS contains 2-substituted, 3-substituted, 2,4-disubstituted and 3,4-disubstituted Rha, 3-substituted ManNAc, terminal Glc, and terminal Fuc3NAc.

The ^{1}H NMR spectra of both OPSs were essentially identical (Fig 1). This finding and the results of the chemical analyses suggested the presence of repeating units of the same structure. Further studies were performed with the OPS from *A. brasilense* Sp246.

The ^{13}C NMR spectrum of the OPS (Fig. 2) showed signals of seven anomeric carbons. Analysis using 2D NMR spectroscopy, including ROESY and ^{1}H,^{13}C HSQC experiments, revealed the presence of two distinct repeating units. One of them was tetrasaccharide **1** (Chart 1), whose structure had been established earlier in the OPS of *A. brasilense* 54.[6] In order to determine the structure of the other repeating unit, Smith degradation of the OPS was performed. As a result, repeating units **1** were destroyed and a polysaccharide (PS) was obtained. A comparison of 1D and 2D NMR spectra of the PS and OPS showed that the remaining repeating unit **2** was not affected by Smith degradation.

The ^{13}C NMR spectrum of the PS (Fig. 3) contained signals for three anomeric carbons at δ 95.1–100.8, two CH_3–C groups (C-6 of Rha and Fuc3NAc) at δ 16.5 and 18.0, one $HOCH_2$–C group (C-6 of ManNAc) at δ 61.4, two nitrogen-bearing carbons (C-2 of ManNAc and C-3 of Fuc3NAc) at δ 50.7 and 52.4, other sugar ring carbons in the region of δ 66.1–80.8 and two *N*-acetyl groups (CH_3 at δ 23.0 and 23.3, CO at δ 175.7 and 176.2). The absence of signals in the region of δ 83–88 that are characteristic of furanosides[11] confirmed the pyranosidic form of all monosaccharide residues. Accordingly, the ^{1}H NMR spectrum of the PS showed signals for three anomeric protons at δ 4.99–5.08, two CH_3–C groups (H-6 of Rha and Fuc3NAc) at δ 1.19 and 1.33, other sugar protons at 3.41–4.71 and two *N*-acetyl groups at δ 2.06 and 2.07.

The ^{1}H and ^{13}C NMR signals for three monosaccharide residues were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC and HMBC experiments (Tables 1 and 2). The TOCSY spectrum

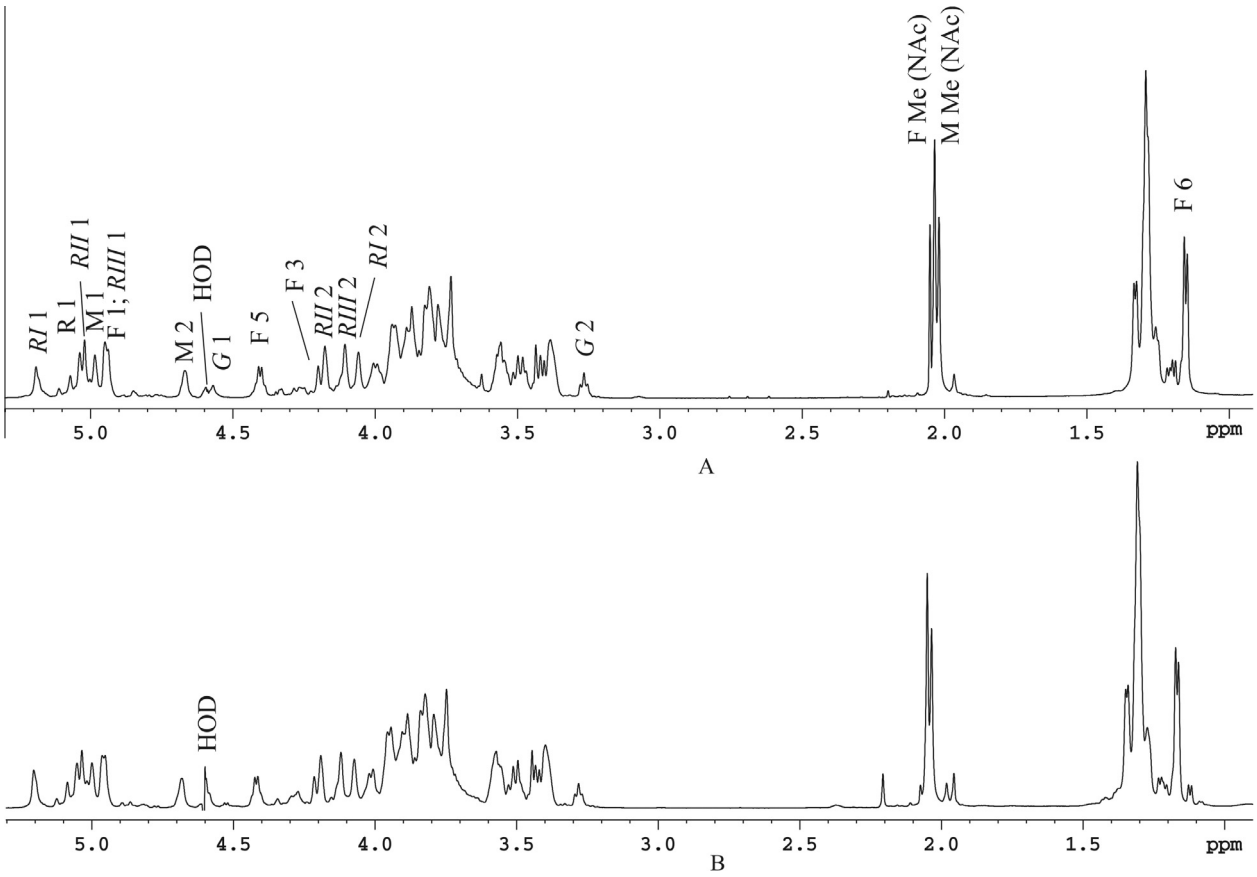

Figure 1. ^{1}H NMR spectra of the OPSs from *A. brasilense* Sp246 (A) and SpBr14 (B). Arabic numerals refer to protons in sugar residues denoted as follows: *G*, Glc; *RI*, Rha^{I}; *RII*, Rha^{II}; *RIII*, Rha^{III} in the repeating unit **1**; R, Rha; M, ManNAc; F, FucNAc in the repeating unit **2**.