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Structural Peculiarities of the O-Specific Polysaccharides of *Azospirillum* Bacteria of Serogroup III

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Abstract—Lipopolysaccharides and O-specific polysaccharides were isolated from the outer membrane of bacterial cells of three strains belonging to two *Azospirillum* species, and their structures were established by monosaccharide analysis including determination of the absolute configurations, methylation analysis, and one- and two-dimensional NMR spectroscopy. It was shown that while having the identical composition, the O-polysaccharides have different branched tetrasaccharide repeating units. Two neutral polysaccharides were found in the lipopolysaccharide of *A. brasilense* 54, and the structure for the predominant O-polysaccharide was determined. The structural data, together with results of serological studies, enabled assignment of strains examined to a novel serogroup, III. The chemical basis for the serological relatedness among the azospirilla of this serogroup is presumably the presence of a common →3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ oligosaccharide motif in their O-polysaccharides.

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Key words: *Azospirillum*, lipopolysaccharide, O-antigen, O-polysaccharide structure, serological characterization

Bacteria of the genus *Azospirillum* are known as ubiquitous phytostimulating microorganisms, which are involved in associative symbiosis with plants [1], in essence mutually beneficial relationships providing competitive advantages to both partners. In the absence of strict host–plant specificity, azospirilla, nonetheless, display selectivity, which becomes apparent already at early stages of recognition and colonization, and surface polysaccharides play an active role in its implementation [2, 3].

Lipopolysaccharide (LPS, O-antigen) is a major, unique for Gram-negative bacteria structural component of the outer membrane of the cell wall. It consists of lipid A, which anchors the molecule in the membrane, and a polysaccharide moiety, which includes a central oligosaccharide (core) and an O-specific polysaccharide (OPS). The structure of the polysaccharide portion of the LPS defines the immunospecificity of the bacterial cell, which is the basis for the classification into a certain serotype or serogroup.

Studies of serological properties and structures (chemotypes) of O-antigens allowed dividing 13 azospirilla strains into two serogroups [4]. OPSs of azospirilla from serogroup I are linear D-rhamnans [5]. These bacteria are widespread in various climate zones and occur usually in association with wheat.

Strains of *Azospirillum* serogroup II that are characterized by the presence of heteropolysaccharide OPSs, cross-react with antibodies against LPS of the reference strain *A. brasilense* Sp7. Comparative studies of the OPS structures revealed certain common fragments, which could be responsible for the immunological relatedness [4].

The hosts for serogroup II azospirilla are a wide range of gramineous plants (crabgrass, cynodon, millet, rice, wheat, etc.). Probably, this fact is responsible for certain heterogeneity of this group of microorganisms, which is

Abbreviations: COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond coherence; HSQC, heteronuclear single quantum coherence; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; OPS, O-specific polysaccharide; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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manifested both in serological properties and in structures of surface polysaccharides. Particularly, the reference strain *A. lipoferum* Sp59b, a representative of serogroup II, is characterized by unique antigen determinants, which are different from those of other strains. Structural analysis of polysaccharide isolated from the outer membrane LPSs and capsular glycopolymers of these bacteria [6, 7] allowed, despite their distinctions, identification of a common α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p* structural fragment, which is rather common in OPSs of phytopathogenic bacteria [8]. Synthesis of this oligosaccharide in the OPS of *Rhizobium* sp. NGR234, which is induced by plant flavonoids, is required for nodulation of leguminous plants and thus mediates the symbiotic interaction [9]. Taking into account the high biological activity of this oligosaccharide fragment, there is an interest in studies of its occurrence in surface polysaccharides of associative bacteria of the *Azospirillum* genus.

The aim of the present study was a serological screening of various strains of *Azospirillum* using antibodies against LPS and capsular polysaccharide of *A. lipoferum* Sp59b and elucidation of OPS structures in serologically related strains.

MATERIALS AND METHODS

Bacterial strains and growth. Bacteria *A. lipoferum* SR66 and SR85 used in the studies were isolated from rhizosphere of wheat in the Saratov Region and came from the collection of microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences (IBPPM, RAS) (Saratov). *Azospirillum brasilense* 54, a microorganism isolated from giant mulberry para-nodules [10], was kindly provided by colleagues from the Institute of Agricultural Microbiology, Ukrainian Academy of Agricultural Sciences (Chernigov, Ukraine). Bacteria were grown on liquid sodium-malate medium supplemented with vitamins [11] at 30°C until the end of the exponential growth phase.

Isolation of lipopolysaccharides and O-polysaccharides. Capsular material was washed away from cell surface by mechanical stirring in tenfold volume of 0.15 M NaCl at ambient temperature for 6 days with daily exchange of the washing solution. LPS was extracted from acetone-dried capsule-free cells (20 g of each strain) by the Westphal water–phenol procedure as described [12]. A high-molecular-mass LPS fraction was isolated from the extract by gel filtration on a column (55 × 1.8 cm, V_0 = 40 ml) with Sepharose CL-4B (Pharmacia, Sweden) in 25 mM NH_4HCO_3 (pH 8.3). The yield of LPS was 1.4, 2.9, and 0.7% of dry cells mass of *A. lipoferum* SR66, SR85, and *A. brasilense* 54, respectively.

LPS of each strain was degraded with 2% AcOH at 100°C for 5 h. A lipid A precipitate was removed by centrifugation, and a water-soluble fraction was fractionated by gel-permeation chromatography on a column of Sephadex G-50 (Pharmacia) in 50 mM pyridine-acetate buffer (pH 4.5), monitoring the elution by a differential refractometer (Knauer, Germany). As a result, OPSs were obtained in yields 29.2, 20.9, and 31.5% of mass of LPS_{SR66}, LPS_{SR85}, and LPS_{54}, respectively.

SDS-PAGE of LPS preparations was performed in 15% gel at I_{const} 30 mA [13]. Components were visualized on gels by silver staining [14].

Double radial immunodiffusion was performed according to the standard procedure [15] in 1% agarose gel. The precipitate was stained with Coomassie blue R-250.

Analytical methods. Carbohydrate, protein, and Kdo contents were determined by conventional colorimetric methods [11]. Fatty acid composition of LPSs was determined by GLC of the methyl esters using a GL-2010 chromatograph (Shimadzu, Japan). The methyl esters were prepared as described [16].

Monosaccharide analysis and determination of the absolute configurations of neutral sugars were performed by GLC of the alditol acetates and acetylated glycosides with (*R*)-2-octanol, respectively. The procedure for sample preparation and experimental conditions were as described in [6].

Methylation of OPSs was performed with CH_3I in dimethylsulfoxide in the presence of sodium methylsulfinylmethanide. The methylated OPSs were hydrolyzed with 2 M CF_3CO_2H (100°C, 2 h), and the partially methylated alditol acetates were analyzed by GLC-MS on a Hewlett-Packard HP 5989 chromatograph (Hewlett-Packard, USA) equipped with a HP-5ms capillary column using a temperature program from 150°C (3 min) to 320°C at the heating rate of 5°C/min.

NMR spectroscopy of OPSs. NMR spectra were recorded on an Avance II 600 spectrometer (Bruker, Germany) in 99.95% D_2O at 27°C (internal standards sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4, δ_H 0 ppm; acetone, δ_C 31.45 ppm). Prior to measurements, samples were freeze-dried twice from 99.9% D_2O. Two-dimensional spectra were recorded using standard Bruker software; the TopSpin 2.1 program was used to acquire and process the data. Mixing time in TOCSY and NOESY experiments was set to 150 and 200 msec, respectively.

RESULTS AND DISCUSSION

A group of *Azospirillum* strains, including of *A. lipoferum* SR65, SR66, SR85 and *A. brasilense* S17 and 54, was selected based on preliminary serological experiments. LPS-containing extracts from those strains cross-reacted with antisera against LPS and capsular polysaccharide of *A. lipoferum* Sp59b. The OPS structures of *A.*

brasilense S17 and *A. lipoferum* SR65 were determined by us earlier, and a trisaccharide fragment composed of L-rhamnose residues was identified as their constituent [17, 18]. Serological cross-reactions of the other strains studied suggested the presence of one or several identical or similar determinants accessible for interaction with antibodies.

LPS_{SR66}, LPS_{SR85}, and LPS_{54} were recovered from the phenol–water extract of acetone-dried bacterial cells of *A. lipoferum* SR66 and SR85 and *A. brasilense* 54, respectively, by gel filtration on Sepharose CL-4B. Typical components of LPS macromolecules were identified in these LPSs, namely carbohydrates (from 30 to 60%, depending on the strain), Kdo, heptose, and 3-hydroxyalkanoic acids.

Silver-stained SDS-PAGE revealed similarities of electrophoretic profiles of all LPS studied with clearly seen heterogeneity in respect to molecular masses, O-polysaccharide-containing S-form LPS molecules being predominant.

The O-polysaccharides (OPS_{SR66}, OPS_{SR85}, and OPS_{54}) were released by mild acid hydrolysis of LPS followed by gel chromatography of water-soluble fraction on Sephadex G-50. GLC analysis of the alditol acetates and acetylated (*R*)-2-octyl glycosides obtained after full acid hydrolysis of the polysaccharides showed that OPS_{SR66} and OPS_{SR85} have the same monosaccharide composition and are composed of L-Rha and D-Glc in the ratio ~2.7 : 1. For OPS_{54}, the ratio was ~3.2 : 1 and, in addition, N-acetylmannosamine (ManNAc) and 3-O-methyl-N-acetylgalactosamine (3-OMeGalNAc) were identified as its components.

Methylation analysis of OPS_{SR85} and OPS_{SR66}, including GLC-MS of the partially methylated alditol acetates, revealed 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, 1,2,3,5-tetra-O-acetyl-4-O-methylrhamnitol, and 1,3,5-tri-O-acetyl-2,4-di-O-methylrhamnitol. The same analysis of OPS_{54} revealed 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, 1,3,4,5-tetra-O-acetyl-2-O-methylrhamnitol, 1,2,5-tri-O-acetyl-3,4-di-O-methylrhamnitol, and 1,3,5-tri-O-acetyl-2,4-di-O-methylrhamnitol, as well as much lower amounts of 1,4,5-tri-O-acetyl-2,3-di-O-methylrhamnitol, 1,5-di-O-acetyl-3,4,6-tri-O-methyl-2-(N-methyl)acetamidogalactitol, and 1,3,4,5-tetra-O-acetyl-6-O-methyl-2-(N-methyl)acetamidomannitol. These data suggested that the repeating units of OPS_{SR85} and OPS_{SR66} are built up of 3-substituted Rha, 2,3-disubstituted Rha at the branching point, and a terminal Glc residue, whereas the main repeating unit of OPS_{54} (discounting minor amino sugars) is composed of residues of 3- and 2-substituted Rha, 3,4-disubstituted Rha at the branching point, and a terminal Glc residue.

The ^{13}C and ^{1}H NMR spectra of the OPS of *A. lipoferum* SR85 and those of the previously studied strain *A. lipoferum* SR65 [18], as well as the spectra of the OPS of *A. lipoferum* SR66 and a previously studied polysaccharide from the capsular polysaccharide-containing complex of the reference strain *A. lipoferum* Sp59b [7], were identical; therefore, their repeating units have the same structures (Table 1). Remarkably, all these OPS structures and that of *A. lipoferum* SpBr17 OPS [19] are very similar. The only difference was the stoichiometric O-acetylation of one of the Rha residues in the OPS of *A. lipoferum* SpBr17, whereas the degree of its acetylation in the capsular polysaccharide of *A. lipoferum* Sp59b and the OPS of *A. lipoferum* SR66 was 60-65%.

The ^{13}C NMR spectrum of OPS_{54} contained signals of different intensities (figure), most likely, owing to the presence of a minor fraction of another OPS, whose structure is not reported in this article.

The main series in the ^{13}C NMR spectrum of OPS_{54} (the figure and Table 2) contained signals for four anomeric carbons at 102.0-104.2 ppm, 16 sugar ring carbons in the region 69.3-80.5, and three methyl groups (C6 of Rha) at 17.9-18.5 ppm. The absence of any signals in the region 82-88 ppm, which are typical of furanosides, showed that all monosaccharide residues are in the pyranose form. Accordingly, the ^{1}H NMR spectrum contained signals of four anomeric protons at 4.62-5.23 ppm, as well as sugar protons at 3.30-4.22 ppm and three methyl groups (H6 of Rha) at 1.32-1.37 ppm. Therefore, OPS_{54} has a tetrasaccharide repeating unit consisting of three Rha residues (Rha^{I}-Rha^{III}) and one Glc residue.

The ^{1}H and ^{13}C NMR spectra of OPS_{54} were assigned using two-dimensional COSY, TOCSY, ROESY, $^{1}H/^{13}C$-HSQC, and HMBC experiments (Table 2). The two-dimensional ROESY experiment revealed cross-peaks between the anomeric protons and protons at the linkage carbons at 4.62/3.80, 5.04/4.03, 4.97/4.09, and 5.23/3.91, which were assigned to H1 Glc/H4 Rha^{III}, H1 Rha^{II}/H3 Rha^{III}, H1 Rha^{III}/H2 Rha^{I}, and H1 Rha^{I}/H3 Rha^{II} interresidue correlations, respectively. These data were in agreement with the substitution pattern determined by the ^{13}C NMR chemical shifts and allowed determination of the monosaccharide sequence in the OPS.

A relatively large $J_{1,2}$ coupling constant of ~7 Hz showed that the Glc residue is β-linked. Relatively low-field positions of the H1, H3, and H5 signals of all rhamnose residues at 4.97-5.23, 3.91-4.03, and 3.76-3.85 ppm, respectively, as well as the C5 chemical shifts of 69.3-70.4 ppm indicated that these residues are α-linked (compare published data for β-Rha*p* and α-Rha*p* [20, 21]).

Based on the data obtained, it was concluded that the repeating unit of the OPS of *A. brasilense* 54 is a branched tetrasaccharide having the structure shown in Table 1.

A comparison of OPS structures in azospirilla, which are serologically related to the reference strain *A. lipoferum* Sp59b, revealed a common →3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ trisaccharide fragment, which, most likely, is responsible for the observed serological cross-reactions. Differences in the activity of heterologous LPS in reactions with antisera against the

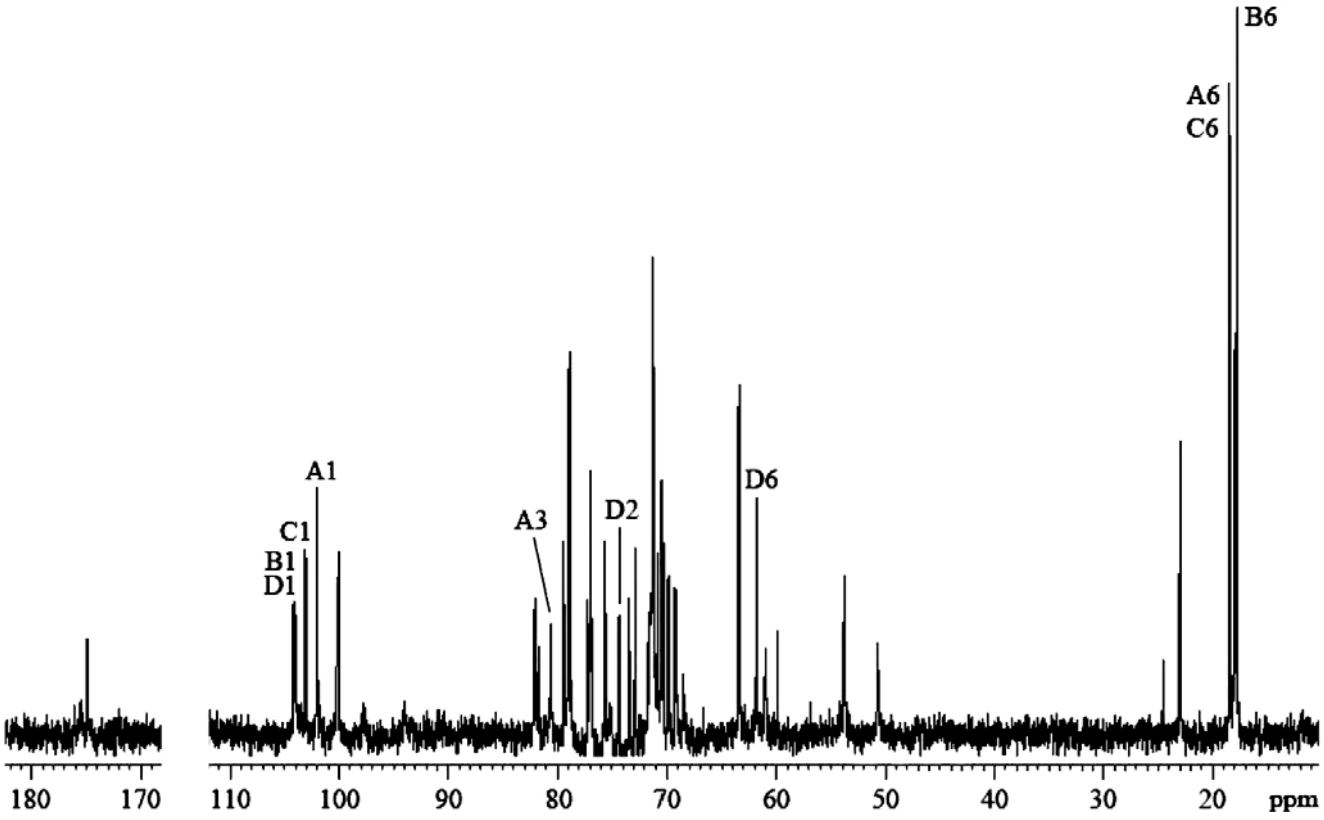
Table 1. Structure of repeating units of the OPSs of *Azospirillum* spp. serogroup III

Strain	Structure of repeating unit	Reference
	A. lipoferum	
Sp59b	α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-β-D-Man*p* 1 ↓ 4 →3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→	[6]
Sp59b* SR66**	β-D-Glc*p* 1 ↓ 3 OAc ~60–65% | 2 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→	[7]
SR65 SR85**	β-D-Glc*p* 1 ↓ 3 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→	[18]
SpBr17	β-D-Glc*p* 1 ↓ 3 OAc | 2 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→	[19]
	A. brasilense	
S17	β-D-Glc*p* 1 ↓ 2 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→	[17]
54**	β-D-Glc*p* 1 ↓ 4 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→	

* Shown is structure of capsular polysaccharide of *A. lipoferum* Sp59b.
** Structure of OPS was determined in this work.

prevalent surface glycopolymers of *A. lipoferum* Sp59b could be accounted for by a different location of the terminal glucose residue and the degree of acetylation of one of the rhamnose residues. In spite of a common architectonics of the oligosaccharide repeating units, the heterogeneity can significantly influence the physiological activity of the O-antigens. It is not excluded that the unique (for azospirilla) ability to induce formation of para-nodules on mulberry roots [10] manifested by *A. brasilense* 54 is related to the peculiar features of the O-antigen structure of this strain.

The data allow the classification *A. lipoferum* Sp59b, SR65, SR66, SR85, SpBr17 and *A. brasilense* S17 and 54 into a new *Azospirillum* serogroup, III. The accumulation



^{13}C NMR spectrum of O-specific polysaccharide of *A. brasilense* 54

of serotyping data, together with LPS-based chemotyping of azospirilla, provides a possibility of monitoring the formation of associative relationships between these microorganisms and plants as well as the elucidation of the topology and functions of bacterial cell surface glycans, including their role in the formation of stable associations efficient for plants.

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Table 2. ^{1}H and ^{13}C NMR data of OPS of *A. brasilense* 54 (chemical shifts, ppm)

Monosaccharide residue	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 (6a; 6b) C6
→2)-α-L-Rha*p*^{I}-(1→ (A)	5.23 102.0	4.09 79.4	3.97 71.2	3.53 73.4	3.85 70.4	1.37 17.9
→3)-α-L-Rha*p*^{II}-(1→ (B)	5.04 104.0	4.22 71.2	3.91 78.9	3.54 72.9	3.76 70.4	1.32 18.5
→3)-α-L-Rha*p*^{III}-(1→ (C) 4 ↑	4.97 103.1	4.14 71.2	4.03 80.5	3.80 78.9	3.84 69.3	1.33 18.4
β-D-Glc*p*-(1→ (D)	4.62 104.2	3.30 74.4	3.46 77.2	3.42 70.7	3.42 76.9	3.77; 3.94 61.8

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