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EXPERIMENTAL ARTICLES

O-Polysaccharide Structure in Serogroup I Azospirilla

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Abstract—Lipopolysaccharides (LPS) and O-specific polysaccharides (OPS) were obtained from the outer membrane of four *Azospirillum* strains previously assigned to serogroup I based on the serological affinity revealed by the antibodies (AB) to the LPS of *A. brasilense* Sp245. Investigation, including determination of monosaccharide composition, methylation analysis, and one- and two-dimensional NMR spectroscopy, was carried out to determine the OPS structure. The OPSs of *A. brasilense* Sp107 and S27 and of *A. lipoferum* RG20a were found to have an identical structure of repeating units represented by a linear penta-D-rhamnan, as was previously described for the OPSs of *A. brasilense* Sp245 and SR75. The OPS of *A. brasilense* SR15 was found to consist of tetrasaccharide repeating units of the following structure: $\longrightarrow$ 2)-α-D-Rha*p*-(1 $\longrightarrow$ 2)-β-D-Rha*p*-(1 $\longrightarrow$ 3)-α-D-Rha*p*-(1 $\longrightarrow$ 2)-α-D-Rha*p*-(1 $\longrightarrow$. An opine compound, N^{δ}-(1-carboxyethyl)-ornithine, closely associated with the LPS of *A. brasilense* SR15, was identified in azospirilla for the first time. The presence of a 6-deoxisugar (D-rhamnose) in the OPS structure was shown to be the chemical basis of the serological similarity and the reason for classification of these strains within the serogroup I.

Key words: Azospirillum, lipopolysaccharide, structure of O-specific polysaccharides, serological characterization.

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Nitrogen-fixing soil bacteria of the genus *Azospirillum* are among the microorganisms stimulating plant growth. They form associations, colonizing the surface (and sometimes internal tissues) of the roots of a broad range of macroscopic partners [1]. Although many aspects of this plant–microbial interaction have been investigated in detail, the molecular mechanism of formation of this association is still insufficiently understood. An important role of glycopolymers [2], as well as of lectin–carbohydrate interactions [3], in the processes of macro-associant recognition and attachment of azospirilla to the root surface has been demonstrated. The activity of lipopolysaccharides (LPS) in azospirilla at the initial stages of formation of the association is poorly studied due to the complex composition of these macromolecules, although their role in cell adsorption on plant roots was established for some strains [4].

In gram-negative bacteria, the cell envelope consists of the inner and outer membranes separated by a thin peptidoglycan layer. The outer membrane contains three-component LPS molecules, which are fixed by the lipid A submerged into the phospholipid layer. The carbohydrate components of the LPS, represented by the core oligosaccharide and O-specific polysaccharide (OPS), are exposed to the outer space. Due to its surface location, LPS may be involved in the processes of symbiont recognition by the macroscopic host and in initiation of the immune response. LPS of several types are produced by bacteria; the S form is characterized by the presence of all the above components, while the R form is lacking the O-specific chain, and in the transitory SR form, OPS is represented by one repeating unit [5]. OPSs exhibit structural variability resulting from a broad range of component monosaccharides and noncarbohydrate substituents and differences in monomer sequence and character of substitution. LPSs are basic heat-stable antigens of the bacterial cell surface and are important for the serological identification of some gram-negative bacteria. The antibodies (AB) to LPS are known to exhibit specificity to the antigen determinants located in the polysaccharide part of the molecule [6, 7]. Our previous serological investigation of nine azospirilla strains suggested their subdivision into two serogroups. The LPSs of the group I azospirilla exhibited a cross reaction at the interaction with the AB to glutaraldehyde-treated *A. brasilense* Sp245 whole cells (AB_{Sp245}) [8]. The structure of *A. brasilense* Sp245 LPS was determined; its repeating unit was shown to be linear penta-D-rhamnan [9]. Since polyclonal AB_{Sp245} were used in the serological experiments, which are

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Table 1. Bacterial strains used in the work

Bacterial Strain		Host plant and strain source	Reference
A. brasilense	SR 15	*Dactilis glomerata* L., isolated in the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russ. Acad. Sci.	[10]
	S27 (ICAR)	*Sericostoma pauciflorum* L., provided by Prof. A.N. Lahiri	No description
	Sp107	*Triticum aestivum* L., provided by Prof. J. Döbereiner	[11]
A. lipoferum RG20a			[12]

formed in response to the whole pool of the LPS antigens, the existence of common structural fragments in the polysaccharide chains of the LPSs of serogroup I bacteria was only suggested.

The goal of the present work is to investigate the OPS structure in azospirilla of serogroup I in order to reveal the chemical basis for their serological relation.

MATERIALS AND METHODS

The azospirilla strains used in the present work are listed in Table 1. All microorganisms were grown in liquid malate–salt medium with vitamins [2] to the end of the exponential growth phase. The capsule was removed from the cell surface by washing in the physiological saline for three days, with daily change of the washing solution. The biomass was dried by centrifugation in acetone.

LPS isolation from dry bacterial biomass (20 g) was carried out with hot 45% aquatic phenol according to a modified Westfal method [13]. Phenol was removed from the extracts by dialysis; they were then concentrated and fractioned by gel filtration on a Sepharose CL-4B column (41 × 1.8 cm, $V_0 = 35$ ml) with 0.025 M NH_4HCO_3 (pH 8.3) as an eluent. The separated products in the eluate were detected using an LKB 2142 differential flow refractometer (LKB, Sweden). Carbohydrate-containing fractions were revealed by the phenol–sulfuric acid reaction. The content of nucleic acids and protein was controlled spectrophotometrically by adsorption at 240–260 and 280 nm, respectively.

OPS isolation was carried out by the standard method of acid degradation (2% CH_3COOH at 100°C, 4–5 h) of the lyophilized LPS preparation. To precipitate the water-insoluble lipid fraction, the hydrolysate was centrifuged for 20 min at 13000 *g*; the supernatant was then fractionated on a Sephadex G-50 column (55 × 2.6 cm, $V_0 = 90$ ml) in 0.05 M pyridine–acetate buffer (pH 4.5). The fractions exiting with the idle column volume were collected and lyophilized.

Analytical techniques. Colorimetric determination of carbohydrates, 2-keto-3-deoxyoctanoic acid (KDO), and phosphorus in the LPS preparations was carried out by the methods described earlier [2]. The measurements were taken on a Specord 40 spectrophotometer (Analytik Jena AG, Germany).

Analysis of the monosaccharide composition of polysaccharides (PS) was carried out on a Hewlett-Packard 5890 gas–liquid chromatograph after sample hydrolysis with 2 M CF_3COOH (120°C, 2 h), reduction with $NaBH_4$, and acetylation [14]. The separation was carried out on an HP-5 capillary column in the temperature gradient from 160°C (1 min) to 290°C with a heating rate of 7°C/min. The absolute configurations of sugars were determined by gas–liquid chromatography of acetylated glycosides with (S)-2-octanol under the same conditions [15].

Methylation of PS was carried out with CH_3I in dimethyl sulfoxide in the presence of methylsulfinylmethanide [16]. PSs were then hydrolyzed with 2 M CF_3COOH (120°C, 2 h) and acetylated after reduction with $NaBH_4$. Analysis of the partially methylated polyol acetates was carried out on a Hewlett-Packard 5989A chromatograph with an HP-5ms capillary column at the temperature gradient from 150°C (3 min) to 320°C with the heating rate of 5°C/min.

The fatty acid (FA) composition in lipid A was analyzed on a Biokhrom 1 gas–liquid chromatograph equipped with a 25-m capillary column with the OV-101 static phase. FA identification was carried out by determination of fatty acid methyl ethers (FAME) as a percent of the total FAME content determined chromatographically. Methylation was carried out as described in the work [17].

Smith degradation was carried out by oxidation of the polysaccharide (40 mg) with 0.1 M $NaIO_4$ in the dark for 72 h at 20°C. After addition of an excess of ethylene glycol, it was reduced with $NaBH_4$ and desalinated in a water solution on the column (80 × 1.6 cm, $V_0 = 45$ ml) with Toyopearl TSK HW-40. The modified OPS was hydrolyzed in 2% CH_3COOH (100°C, 2 h). The degradation products were separated on a column with Toyopearl TSK HW-40.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for OPS solutions in D_2O at 27°C. The chemical shifts were assayed with 3-trimethylsilylpropionate-d_4 (δ_H 0.0) and acetone (δ_C 31.45) as internal standards.

Inhibition of LPS interaction with AB_{Sp245} monosaccharides and OPS was determined by the dual radial immunodiffusion test [18] and by the solid-phase enzyme immunoassay (EIA). AB_{Sp245} were treated by water solutions of mono- and polysaccharides (further on termed inhibitors) for 2 h. For immunodiffusion, the LPS solutions (0.5 mg/ml) were applied to the peripheral wells and titrated; AB_{Sp245} incubated with the inhibitors or intact (the control) were applied to the central wells. The gels were stained with Coomassie R-250. Inhibition was determined

Table 2. Chemical composition of the LPS

Components, content, %		LPS_{SR15}	LPS_{S27}	LPS_{RG20a}	LPS_{Sp107}
Carbohydrates		28.5 ± 1.8	25.3 ± 3.2	37.4 ± 2.1	50.9 ± 3.4
KDO		2.8 ± 0.3	2.7 ± 0.1	2.3 ± 0.1	1.3 ± 0.4
Total phosphorus		1.4 ± 0.2	0	0	0
FAME, %	3-hydroxytetradecanoic acid	23.8 ± 0.3	24.9 ± 0.4	54.4 ± 1.5	32.4 ± 0.7
	hexadecanoic acid	4.7 ± 0.1	8.8 ± 0.2	3.2 ± 0.7	0.6 ± 0.1
	3-hydroxyhexadecanoic acid	15.3 ± 0.2	16.7 ± 0.1	36.4 ± 0.6	15.4 ± 1.0
	octadecenoic acid	22.7 ± 0.2	5.9	4.7 ± 0.3	19.0 ± 2.2

Note: The results are presented as mean values with standard deviations; the confidence interval is given for 95% reliability.

visually as the absence of precipitation bands between the LPS and AB_{Sp245}.

For EIA, LPS was incubated in 96-well trays (100 ng per well, 2 h at 4°C) and then supplemented with AB_{Sp245}. The bound AB_{Sp245} were revealed by the AB conjugate to rabbit immunoglobulin G with horseradish peroxidase. Hydrogen peroxide with *o*-phenylenediamine was used as the substrate reagent. The optical density of the samples was measured at 490 nm on an AIF-Ts-01S immune enzyme analyzer (ILIP, St. Petersburg, Russia).

RESULTS AND DISCUSSION

In the present work, the LPSs of *A. brasilense* Sp107, SR15, and S27 and of *A. lipoferum* RG20a were isolated with a yield of 1.1–2.5% of the dry biomass weight. All the characteristic LPS components (carbohydrates, KDO, and lipid A FA) were revealed in the preparations (Table 2). The LPSs differed in carbohydrate content, which decreased in the row LPS_{Sp107}–LPS_{RG20a}–LPS_{SR15}–LPS_{S27}. Their higher content in the LPS_{Sp107} and LPS_{RG20a} preparations may result from predominance of the S forms of the molecules, as confirmed by electrophoretic separation of the LPS preparations (Fig. 1). Electrophoresis revealed that the LPSs of all strains were heterogeneous by molecular mass and represented by both S and R forms, although in *A. brasilense* Sp107 and *A. lipoferum* RG20a the molecules predominated with high degrees of substitution of the core oligosaccharide by polysaccharide chains.

Only one strain, *A. brasilense* SR15, contained phosphorus in its LPS. Phosphate groups are usually located in the lipid core part of the LPS molecule. The structure of lipid A in *A. lipoferum* SpBr17 was recently established, in which phosphate substituents in the characteristic glucosaminebiose 1 and 4' positions were missing [19]. Since within a bacterial genus the hydrophobic LPS component is the most conservative part of the macromolecule, the lipids A of the strains under study probably have the same structure. The absence of phosphorus in the repeating unit of OPS_{SR15} (see below) suggests that individual monosaccharides of the core oligosaccharide of LPS_{SR15} are phosphorylated.

Gas–liquid chromatography of FAME of the lipids A of the azospirilla strains revealed predominance of 3-hydroxytetradecanoic, hexadecanoic, 3-hydroxyhexadecanoic, and octadecenoic acids, although their ratios varied significantly for different strains (Table 2). For example, in *A. brasilense* Sp107 lipid A, trace amounts of hexadecanoic acid were found (less

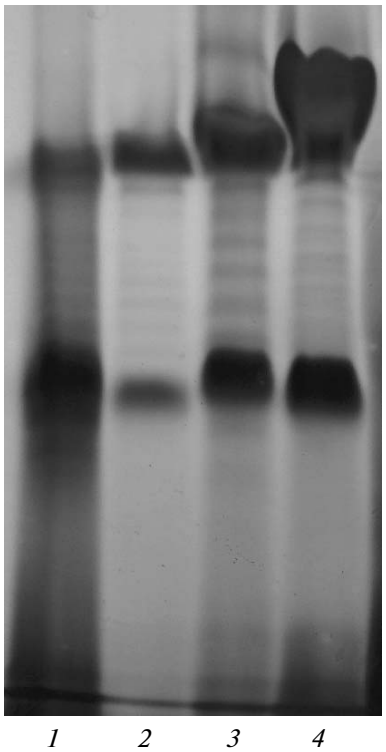

Fig. 1. SDS electrophoresis in PAAG of the LPS preparations from *A. brasilense* SR15 (*1*), *A. brasilense* S27 (*2*), *A. lipoferum* RG20a (*3*), and *A. brasilense* Sp107 (*4*).

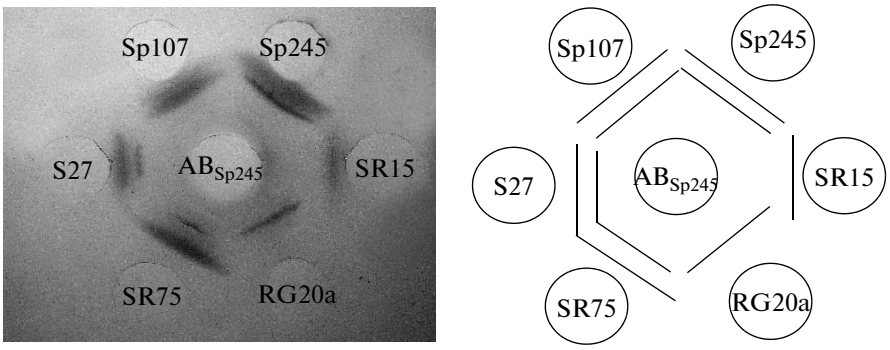

Fig. 2. Dual radial immunodiffusion of the preparations of LPS_{Sp107}, LPS_{Sp245}, LPS_{SR15}, LPS_{S27}, LPS_{SR75}, and LPS_{RG20a} with the antibodies to glutaraldehyde-treated whole cells of *A. brasilense* Sp245 (AB_{Sp245}).

than 1%), while its content in LPS_{S27} was about 9%. The highest content of octadecenoic acid was found in LPS_{Sp107} and LPS_{SR15}, while the highest content of hydroxy acids was in LPS_{RG20a}. The structure of lipid A is known to be based of the carbohydrate skeleton of two D-glucosamine residues, to which hydroxy acid residues are bound by amide and ether bonds. Other FA may be attached to the 3-OH groups of hydroxy acids by ether bonds. For the lipid A of *A. lipoferum* SpBr17, 3-hydroxyhexadecanoic acid was found to be attached to glucosaminebiose by amide bonds, while 3-hydroxytetradecanoic acid is attached by ether bonds; they may be etherified with hexadecanoic and octadecenoic FA [19]. The results presented in Table 2 suggest that predominant acyl residues bound to 3-hydroxyhexadecanoic acid were octadecenoic acid for LPS_{Sp107} and LPS_{SR15} and hexadecanoic acid for LPS_{S27}. Hydroxydidecanoic, decanoic, hexadecenoic, and nanodecanoic acids, which were also found in the lipids A, may also act as 3-hydroxy acid substitutes. However, since their summary content did not exceed 6–7% of the total FAME, these FA are probably components of the phospholipids, which may be extracted from the membrane together with LPS. Considering the ubiquitous distribution of azospirilla, the presence of unsaturated FA in the hydrophobic part of their LPS results probably from the action of a compensatory mechanism maintaining the phase state of the membrane [20]. This state is possibly responsible for its optimal fluidity and enables better adaptation of the cells to changing ambient temperature.

Double radial immunodiffusion of the LPSs of *A. brasilense* Sp107 (LPS_{Sp107}) and *A. brasilense* S27 (LPS_{S27}) with AB_{Sp245} revealed the existence of the serological cross reactions with the LPS of *A. brasilense* Sp245, the reason these strains were assigned to serogroup I (Fig. 2). We have previously demonstrated that *A. brasilense* Sp245 and SR75 are serologically identical and have the same OPS structure [21]. The cross reaction for only one of the precipitation bands for the LPSs of strains RG20a and SR15 (LPS_{RG20a} and LPS_{SR15}) with AB_{Sp245} may result either from the lower exposure of the second epitope or from some differences in the structure of antigenic determinants. In order to determine these differences, OPSs of the above organisms were isolated and investigated.

The OPS preparations were obtained by mild acidic hydrolysis of the LPS with subsequent precipitation of lipid A and gel fractionation of the water-soluble fractions. Their yield was 24–30% of the LPS mass. Determination of their monosaccharide composition and absolute configuration of the sugars by gas–liquid chromatography revealed that all the OPSs were D-rhamnans.

Chromatography–mass spectrometry of the partially methylated polyol acetates revealed the presence of 3,4-di-O-methyl-6-deoxyhexose and 2,4-di-O-methyl-6-deoxyhexose in a ratio of ~ 3 : 1 for the polysaccharide of *A. brasilense* SR15 and of ~ 3 : 2 for the OPSs of three other azospirilla strains. These data suggested the linear structure of all the OPSs studied. Mass spectra of the OPS revealed minor amounts of other monosaccharides (hexoses and deoxyhexoses, once- and twice-substituted). However, the intensity of the corresponding signals was 20–40 times lower than for the main series.

The complete OPS structure was determined by NMR spectroscopy. The ^{13}C-NMR spectra of the polysaccharides of OPS_{Sp107}, OPS_{S27}, and OPS_{RG20a} (Fig. 3) were identical to each other and to the spectra of previously investigated OPS_{Sp245} and OPS_{SR75} [9, 21]. They contained the signals of methyl groups (Rha C-6) of five Rha residues at 17.8–18.2 ppm, five anomeric carbon atoms (Rha C-1) at 98.1–103.6 ppm, and 20 signals of the secondary carbon atoms of monosaccharide cycles at 68.7–79.8 ppm. The ^{1}H-NMR spectra of these polymers were also identical and contained the signals of five methyl groups (Rha H-6) at 1.27–1.32 ppm, five anomeric protons (Rha H-1) at 4.82–5.20 ppm, and other protons at 3.41–4.25 ppm. These data confirm that OPS_{Sp107}, OPS_{S27},

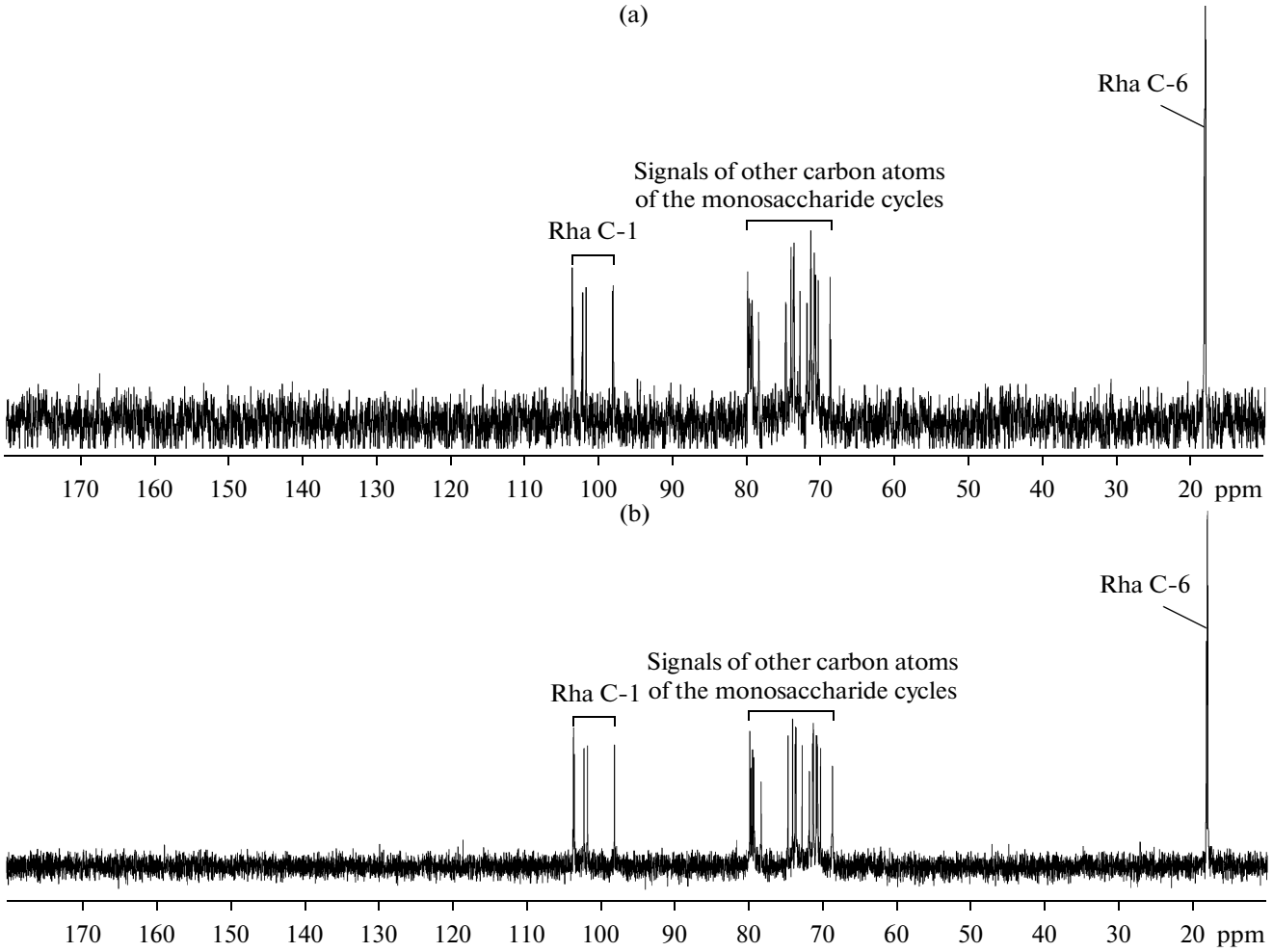

Fig. 3. ^{13}C-NMR spectra of O-specific polysaccharides of *A. lipoferum* RG20a (a) and A. brasilense S27 (b).

and OPS_{RG20a} are built from the pentasaccharide repeating units of the same structure, which contain only the D-Rha residues:

$$\longrightarrow 2)\text{-}\beta\text{-D-Rha}p\text{-}(1 \longrightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \longrightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \longrightarrow 2)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \longrightarrow 2)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \longrightarrow .$$

The ^{13}C-NMR spectrum of the polysaccharide of *A. brasilense* SR15 differed from the above ones (Fig. 4) and contained the signals of four anomeric carbon atoms (Rha C-1) at 98.3–103.3 ppm, four methyl groups (Rha C-6) at 17.8–17.9 ppm, and the other 16 carbon atoms of the monosaccharide cycles at 68.8–79.8 ppm. In the ^{1}H-NMR spectrum of OPS_{SR15}, the signals of four anomeric protons (Rha H-1) at 4.79–5.19 ppm, four methyl groups (Rha H-6) at 1.28–1.32 ppm, and of other monosaccharide protons at 3.44–4.22 ppm were present.

These data demonstrated that OPS_{SR15} is a D-rhamnan with a tetrasaccharide repeating unit. The location of C-6 signals at about 18 ppm and the absence of the signals below 80 ppm in the ^{13}C-NMR spectrum indicates that all the D-Rha residues in the OPS are in the pyranose form [22].

The ^{1}H- and ^{13}C-NMR spectra of the polysaccharide were deciphered (Table 3) using two-dimensional spectroscopy (COSY, TOCSY, NOESY, ROESY, and ^{1}H, ^{13}C HSQC). Location of the H-1,3,5 signal for one of the Rha residues (Rha^{II}) in a relatively high field at 4.79, 3.69, and 3.44 ppm, respectively, demonstrated that this residue is β-bound, while three other Rha residues (Rha^{I}, $Rha^{III–IV}$), where the H-1,3,5 protons resonated at a lower field at 5.02–5.19, 3.90–3.98, and 3.72–3.73 ppm, respectively, are α-bound, in accordance with the published data for β- and α-Rha*p* [23]. The effects of glycosylation on the chemical shifts of rhamnose residues observed in ^{13}C-NMR spectra comply with the results of investigation of the monosaccharide absolute configurations and configurations of the glycoside bonds [24].

The NOESY experiment revealed the interlink correlations between anomeric and bound protons: Rha^{I} H-1/Rha^{II} H-2, Rha^{II} H-1/Rha^{III} H-2 and H-3, Rha^{III} H-1/Rha^{IV} H-2 and Rha^{IV} H-1/Rha^{I} H-2 at δ

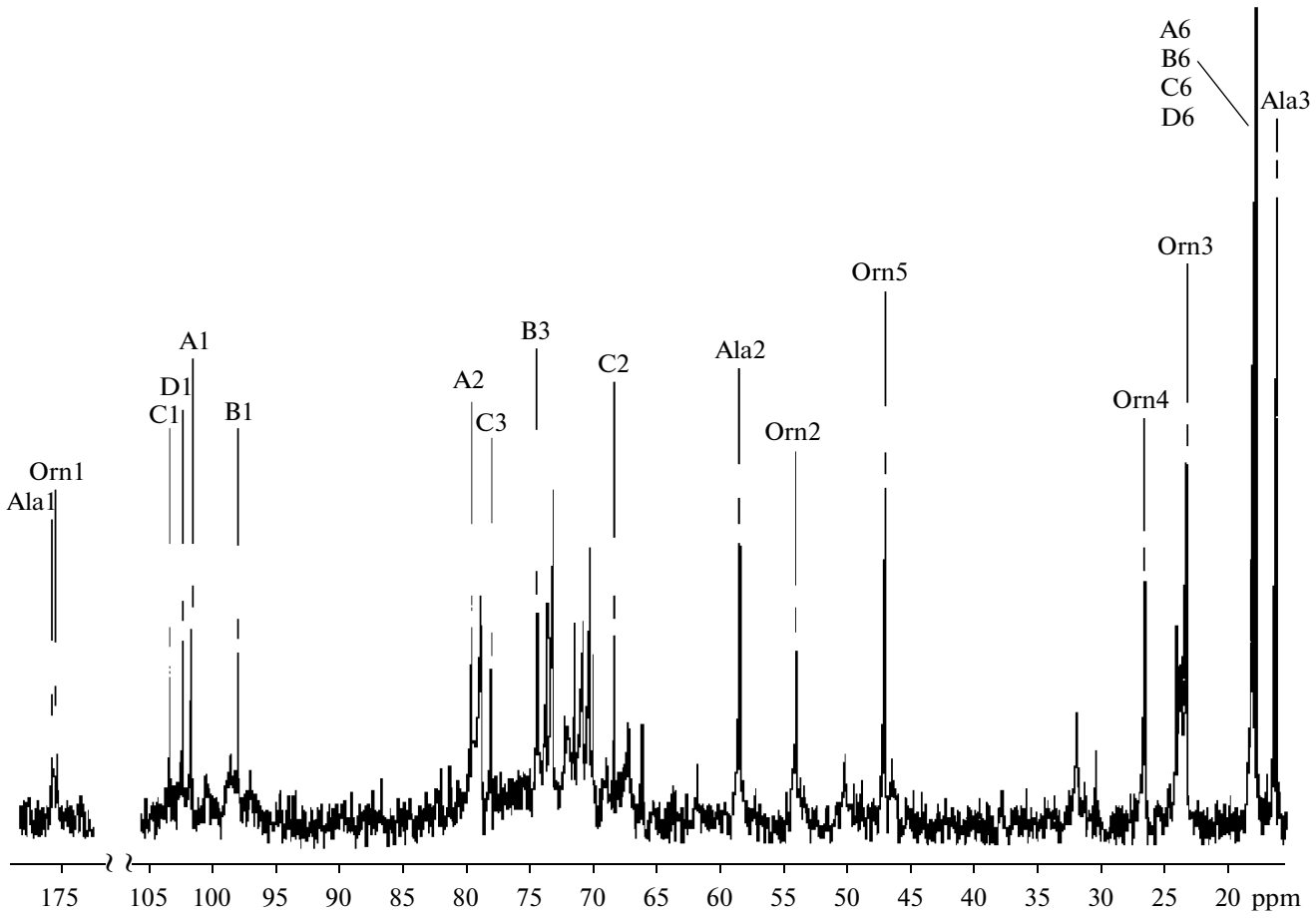

Fig. 4. ^{13}C-NMR spectrum of O-specific polysaccharide of *A. brasilense* SR15.

5.19/4.08, 4.79/3.69 and 3.94, 5.02/4.08, and 5.09/4.08, respectively. Rha^{II} was characterized by the H-1, H-2,3,5 interlink correlations, while the other three Rha residues exhibited only H-1, H-2 correlations. These data confirmed the substitution positions and anomeric configurations of the monosaccharides and enabled us to determine their sequence in the repeating unit of the polysaccharide.

Thus, the repeating unit of *A. brasilense* SR15 OPS has the following structure:

$$\longrightarrow 2)\text{-}\alpha\text{-D-Rha}p^{I}\text{-}(1 \longrightarrow 2)\text{-}\beta\text{-D-Rha}p^{II}\text{-}(1 \longrightarrow 3)\text{-}\alpha\text{-D-Rha}p^{III}\text{-}(1 \longrightarrow 2)\text{-}\alpha\text{-D-Rha}p^{IV}\text{-}(1 \longrightarrow .$$

Apart from the signals from monosaccharide residues, in the ^{1}H-NMR spectrum of OPS_{SR15} two spin systems were determined, corresponding to the ornithine (Orn) and alanine (Ala) residues. In the ^{13}C-NMR spectrum (Fig. 4), all the signals related to these amino acid residues were also present. However, the signals of C-5 Orn residue and C-2 of Ala residue were significantly shifted to the weak field, to 47.2 and 58.6 ppm, relative to their position at 45.4 and 57.8 ppm, respectively, in the spectra of unsubstituted amino acids (Table 3). These data suggested identification of this compound as N^{δ}-1-(carboxyethyl)-ornithine, which, to our knowledge, have not been previously found in bacterial LPS complexes. Its presence may explain the high "protein" concentration (5.4%) revealed in LPS_{SR15} by the Bradford method. To reveal the nature of the peptide compound, Smith degradation was carried out. Since only the monosaccharide residues containing adjacent hydroxyl groups in the ring are subject to periodate oxidation, the polysaccharide of strain *A. brasilense* SR15 degraded almost completely. We suggested that the polypeptide admixture is a polymer and analyzed the minor high-molecular fraction (3.5 mg). However, NMR spectroscopy revealed that this fraction consisted of the OPS residues that did not undergo complete hydrolysis, while amino acid signals were completely absent from the NMR spectra. Thus, N^{δ}-(1-carboxyethyl)-ornithine is not polymerized. It is probably relatively closely associated with OPS_{SR15} due to some physicochemical interactions; in any case, this compound is certainly not a structural component of the repeating OPS unit.

N^{δ}-(1-carboxyethyl)-ornithine was originally identified in the intracellular amino acid pool of lactic acid bacteria *Lactococcus* (*Streptococcus*) *lactis* 133 [25].

Table 3. Data of the ^{1}H-NMR and ^{13}C-NMR spectra of *A. brasilense* SR15 LPS (chemical shifts in ppm)

Monosaccharide residue	H-1	H-2	H-3	H-4	H-5	H-6
	C-1	C-2	C-3	C-4	C-5	C-6
O-polysaccharide						
⟶ 2)-α-D-Rha*p*^{I}-(1 ⟶ (A)	5.19	4.09	3.98	3.41	3.73	1.28
	101.6	79.8	71.6	74.0	70.7	17.8*
⟶ 2)-β-D-Rha*p*^{II}-(1 ⟶ (B)	4.79	4.08	3.69	3.44	3.44	1.32
	98.3	79.0	74.9	74.1	73.9	17.9*
⟶ 3)-α-D-Rha*p*^{III}-(1 ⟶ (C)	5.02	4.22	3.94	3.59	3.72	1.28
	103.3	68.8	78.8	72.0	70.7	17.8*
⟶ 2)-α-D-Rha*p*^{IV}-(1 ⟶ (D)	5.09	4.08	3.90	3.41	3.72	1.28
	102.1	79.4	71.5	74.1	70.7	17.8*
N^{δ}-(1-carboxyethyl)-ornithine						
Ala		3.70	1.48			
	175.9*	58.6	16.2			
Orn		4.38	1.44	1.71	3.03	
	175.7*	54.1	23.3	26.6	47.2	
Amino acid	C-1	C-2	C-3	C-4	C-5	
Ala	174.1	57.8	14.9			
Orn	174.7	54.1	27.6	21.9	45.4	

* Attribution may be reverse.

Its isomer, N^{α}-1-(carboxyethyl)-ornithine (octopinic acid), is produced by plant tumors in response to the presence of the phytopathogenic *Agrobacterium tumefaciens* [26]. The presence of the closest homologue of N^{δ}-(1-carboxyethyl)-ornithine, N^{ε}-(1-carboxyethyl)-lysine was reported for the OPS of some *Providencia* and *Proteus* members [27, 28].

Our experiments demonstrated that the OPSs of all investigated azospirilla strains are linear polymers of D-Rha. The *A. brasilense* SR15 OPS, with the LPS exhibiting the least pronounced capacity for precipitation with AB_{Sp245}, differs from the OPSs of other serogroup I members in the absence of one 3-substituted α-D-Rha residue in the repeating unit. A significant amount of data have been accumulated concerning the presence of D-Rha in the surface glycans of soil microorganisms interacting with plants [8, 29, 30].

To confirm the role of D-Rha in formation of the structure of immunodominant antigenic determinants on the cell surface of serogroup I azospirilla, experiments were carried out on the inhibition of the interaction between AB_{Sp245} and LPS_{SR15}, LPS_{Sp245}, LPS_{SR75}, and LPS_{RG20}. Commercial preparations of D-Gal and L-Rha, as well as D-Rha (O.N. Smol'kina, unpublished data), OPS_{RG20a}, and the previously obtained OPS_{Sp245} and OPS_{SR75} [9, 21], were used as inhibitors. Dual radial immunodiffusion and IEA tests confirmed that OPSs and D-Rha had an inhibitory effect, while L-Rha and D-Gal did not inhibit precipitation. For example, OPS inhibited the LPS/AB_{Sp245} interaction at 0.3 to 1.25 mg/ml for different strains, while D-Rha was active at 3.3 mg/ml.

Thus, our results revealed the serological and structural correlation between the O-antigens of the investigated strains and suggest the major role of D-Rha in formation of immunodominant epitopes of the serogroup I azospirilla.

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REFERENCES

1. Steenhoudt, O. and Vanderleyden, J., *Azospirillum*, a Free Living Nitrogen-Fixing Bacterium Closely Associated with Grasses: Genetic, Biochemical and Ecological Aspects, *FEMS Microbiol. Rev.*, 2000, vol. 24, pp. 487–506.
2. Konnova, S.A., Makarov, O.E., Skvortsov, I.M., and Ignatov, V.V., Isolation, Fractionation and Some Properties of Polysaccharides Produced in a Bound Form by *Azospirillum brasilense* and Their Possible Involvement in *Azospirillum*-Wheat Root Interactions, *FEMS Microbiol. Letts.*, 1994, vol. 118, no. 2, pp. 93–99.
3. Yegorenkova, I.V., Konnova, S.A., Sachuk, V.N., and Ignatov, V.V., *Azospirillum brasilense* Colonization of Wheat Roots and the Role of Lectin-Carbohydrate Interactions in Bacterial Adsorption and Root-Hair Deformation, *Plant Soil*, 2001, vol. 231, no. 2, pp. 975–983.
4. Fedonenko, Yu.P., Egorenkova, I.V., Konnova, S.A., and Ignatov, V.V., Involvement of the Lipopolysaccharides of Azospirilla in the Interaction with Wheat Seedling Roots, *Mikrobiologiya*, 2001, vol. 70, no. 3, pp. 384–390 [*Microbiology* (Engl. Transl.), vol. 70, no. 3, pp. 329–334].
5. Caroff, M. and Karibian, D., Structure of Bacterial Lipopolysaccharides, *Carbohydr. Res.*, 2003, vol. 338, pp. 2431–2447.
6. Weintraub, A., Immunology of Bacterial Polysaccharides Antigens, *Carbohydr. Res.*, 2003, vol. 338, pp. 2539–2547.
7. Poxton, I.R., Antibodies to Lipopolysaccharide, *J. Immunol. Methods*, 1995, vol. 186, pp. 1–15.
8. Konnova, O.N., Boiko, A.S., Burygin, G.L., Fedonenko, Yu.P., Matora, L.Yu., Konnova, S.A., and Ignatov, V.V., Chemical and Serological Studies of Liposaccharides of Bacteria of the Genus *Azospirillum, Mikrobiologiya*, 2008, vol. 77, no. 3, pp. 350–357 [*Microbiology* (Engl. Transl.), vol. 77, no. 3, pp. 305–312].
9. Fedonenko, Yu.P., Zatonsky, G.V., Konnova, S.A., Zdorovenko, E.L., and Ignatov, V.V., Structure of the O-Specific Polysaccharide of the Lipopolysaccharide of *Azospirillum brasilense* Sp245, *Carbohydr. Res.*, 2002, vol. 337, pp. 869–872.
10. Pozdnyakova, L.I., Kanevskaya, S.V., Levanova, G.F., Barysheva, N.N., Pilipenko, T.Yu., Bogatyrev, V.A., and Fedorova, L.S., Taxonomic Study of Azospirilla Isolated from the Cereals of Saratov Oblast, *Mikrobiologiya*, 1988, vol. 57, no. 2, pp. 275–278.
11. Tarrand, J.J., Krieg, N.R., and Döbereiner, J., A Taxonomic Study of the *Spirillum lipoferum* Group, with Descriptions of a New Genus, *Azospirillum* gen. nov. and Two Species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov, *Can. J. Microbiol.*, 1978, vol. 24, no. 8, pp. 967–980.
12. Baldani, V.L.D., Baldani, J.I., and Döbereiner, J., Effect of *Azospirillum* Inoculation on Root Infection and Nitrogen Incorporation in Wheat, *Can. J. Microbiol.*, 1983, vol. 29, no. 8, pp. 924–929.
13. Kul'shin, V.A., Yakovlev, A.P., Avaeva, S.N., and Dmitriev, B.A., Improved Method for Polysaccharide Isolation from Gram-Negative Bacteria, *Mol. Genetika, Mikrobiologiya, Virusologiya*, 1987, no. 5, pp. 44–46.
14. Sawardeker, J.S., Sloneker, J.H., and Jeanes, A., Quantitative Determination of Monosaccharides as Their Alditol Acetates by Gas Liquid Chromatography, *Anal. Chem.*, 1965, vol. 37, pp. 1602–1603.
15. Leontein, K., Lindberg, B., and Lönngren, J., Assigment of Absolute Configuration of Sugars by G.L.C. of Their Acetylated Glycosides Formed from Chiral Alcohols, *Carbohydr. Res.*, 1978, vol. 62, pp. 359–362.
16. Hakomori, S.-I., A Rapid Permetylation of Glycolipids and Polysaccharides Catalyzed by Methylsulfinyl Carbanion in Dimethylsulfoxide, *J. Biochem.*, 1964, vol. 55, pp. 205–208.
17. Mayer, H., Tharanathan, R.N., and Weckesser, J., Analysis of Lipopolysaccharides of Gram-Negative Bacteria, *Methods Microbiol.*, 1985, vol. 18, pp. 157–207.
18. Ouchterlony, O. and Nilsson, L.-A., *Handbook Experimental Immunology*, Weiz, D.M., Ed., Oxford: Alden, 1979, pp. 19–33.
19. Choma, A. and Komaniecka, I., Characterization of Novel Lipid A Structure Isolated from *Azospirillum lipoferum* Lipopolysaccharide, *Carbohydr. Res.*, 2008, vol. 343, pp. 799–804.
20. Solov'eva, T.F. and Ovodov, Yu.S., Physical Characteristics of Gram-Negative Bacteria, *Biol. Membr.*, 1992, vol. 9, no. 3, pp. 245–258.
21. Fedonenko, Yu.P., Borisov, I.V., Konnova, O.N., Zdorovenko, E.L., Katsy, E.I., Konnova, S.A., and Ignatov, V.V., Determination of the Structure of the Repeated Unit of the *Azospirillum brasilense* SR75 O-Specific Polysaccharide and Homology of the *lps* Loci in the Plasmids of *Azospirillum brasilense* strains SR75 and Sp245, *Mikrobiologiya*, 2005, vol. 74, no. 5, pp. 626–632 [*Microbiology* (Engl. Transl.), vol. 74, no. 5, pp. 542–548].
22. Bock, K. and Pedersen, C., Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Monosaccharides, *Adv. Carbohydr. Chem. Biochem.*, 1983, vol. 41, pp. 27–66.
23. Jansson, P.E., Keene, L., and Widmalm, G., Computer-Assisted Structural Analysis of Polysaccharides with an Extended Version of CASPER Using ^{1}H- and ^{13}C-N.M.R. Data, *Carbohydr. Res.*, 1989, vol. 188, pp. 169–191.
24. Shashkov, A.S., Lipkind, G.M., Knirel, Y.A., and Kochetkov, N.K., Stereochemical Factors Determining the Effects of Glycosylation on the ^{13}C Chemical Shifts in Carbohydrates, *Magn. Reson. Chem.*, 1988, vol. 26, pp. 735–747.
25. Thomson, J., Curtis, M.A., and Miller, S.P.F., N^5-(1-Carboxyethyl)-Ornithine a New Amino Acid from the Intracellular Pool of *Streptococcus lactis, J. Bacteriol.*, 1986, vol. 167, no. 2, pp. 522–529.
26. Hack, E. and Kemp, J.D., Comparison of Octopine, Histopine, Lysopine, and Octopinic Acid Synthesizing Activities in Sunflower Crown Gall Tissues, *Biochem. Biophys. Res. Commun.*, 1977, vol. 78, pp. 785–791.
27. Kocharova, N.A., Shashkov, A.S., Shcherbakova, O.V., Knirel', Yu.A., Kochetkov, N.K., Kholodkova, E.V., and Stanislavskii, E.S., Structure of the O-Specific

Polysaccharide of *Providencia alcalifaciens* O23 Containing a New component, Glucuronic Acid Amide with N^{ε}-(1-carboxyethyl)-lysine, *Biokhimiya*, 1997, vol. 62, no. 5, pp. 591–599 .

28. Kondakova, A.N., Toukach, F.V., Senchenkova, S.N., Arbatskii, N.P., Shashkov, A.S., Knirel', Yu.A., Bartodziiska, B., Chikh, K., Rozhal'ski, A., and Sidorchik, Z., New Structures of the O-Specific Polysaccharides of *Proteus*. 3. Polysaccharides Containing Non-carbohydrate Organic Acids, *Biokhimiya*, 2003, vol. 68, no. 4, pp. 548–561 [*Biochemistry (Moscow)* (Engl. Transl.), vol. 68, no. 4, pp. 446–459].

29. Ovod, V., Rudolph, K., Knirel, Y., and Krohn, K., Immunochemical Characterization of O Polysaccharides Composing the Alpha-D-Rhamnose Backbone of Lipopolysaccharide of *Pseudomonas syringae* and Classification of Bacteria Into Serogroups O1 and O2 with Monoclonal Antibodies, *J. Bacteriol.*, 1996, vol. 178, pp. 6459–6465.

30. Zdorovenko, G.M., Zdorovenko, E.L., and Varbanets, L.D., Composition, Structure, and Biological Properties of Lipopolysaccharides from Different Strains of *Pseudomonas syringae* pv. *atrofaciens, Mikrobiologiya*, 2007, vol. 76, no. 6, pp. 774–789 [*Microbiology* (Engl. Transl.), vol. 76, no. 6, pp. 683–697].