ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 5, pp. 606-613. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © O. N. Smol'kina, V. V. Kachala, Yu. P. Fedonenko, G. L. Burygin, E. L. Zdorovenko, L. Yu. Matora, S. A. Konnova, V. V. Ignatov, 2010, published in Biokhimiya, 2010, Vol. 75, No. 5, pp. 707-716.

Capsular Polysaccharide of the Bacterium *Azospirillum lipoferum* Sp59b: Structure and Antigenic Specificity

O. N. Smol'kina[1]*, V. V. Kachala[2], Yu. P. Fedonenko[1], G. L. Burygin[1], E. L. Zdorovenko[2], L. Yu. Matora[1], S. A. Konnova[1,3], and V. V. Ignatov[1]

[1]*Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, 410049 Saratov, Russia; fax: (8452) 970-383; E-mail: room308@ibppm.sgu.ru*
[2]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 119991 Moscow, Russia; E-mail: evelina@ioc.ac.ru*
[3]*Saratov State University, ul. Astrakhanskaya 83, 410012 Saratov, Russia; E-mail: konnovasa@info.sgu.ru*

Received May 7, 2009
Revision received June 16, 2009

Abstract—Antigenic differences were revealed between the cell wall outer membrane lipopolysaccharides and the capsular high molecular weight bioglycans for a typical strain of the nitrogen-fixing rhizobacterium *Azospirillum lipoferum* Sp59b using antibodies prepared against the homologous lipopolysaccharide and lipopolysaccharide–protein complex. From the capsular lipopolysaccharide–protein and polysaccharide–lipid complexes of *A. lipoferum* Sp59b, polysaccharides were isolated and their structure was for the first time established in *Azospirillum* by monosaccharide analysis which included determination of the absolute configurations, methylation, *O*-deacetylation, and one- and two-dimensional NMR spectroscopy. The polysaccharides of the capsular complexes were shown to have identical structure of the branched tetrasaccharide repeating unit, which differs from the structure of the O-specific polysaccharide within the outer membrane lipopolysaccharide of this strain.

DOI: 10.1134/S000629791005010X

Key words: *Azospirillum*, capsular antigens, polysaccharide structure

During recent years, Gram-negative soil diazotrophic bacteria of the *Azospirillum* genus have been actively used as a model for studies on regularities of production and functioning of plant–microbial associations. Although the molecular mechanism of this interaction is not known in detail, it is known that attachment of bacteria to plant roots is a necessary stage in formation of an active association. This process significantly involves polysaccharides located on the surface of bacterial cells [1, 2]. Lipopolysaccharides and capsular polysaccharides (CPS) are the main glycopolymers of *Azospirillum*. Lipopolysaccharide (LPS, O-antigen) is a prevalent (obligatory) component of the cell wall outer membrane of Gram-negative bacteria and consists of three structurally different components: lipid A, core oligosaccharide, and O-specific polysaccharide (OPS). Some bacteria can release an exocellular LPS [3]. O-Antigens play an important role in the interrelationships of the bacterial cell with the environment. Under certain conditions of cultivation of microorganisms, a capsule is produced on the cell surface, which is a self-dependent polyfunctional organoid of a bacterial cell consisting of proteins and CPS. These polymers can induce an immune response (K-antigens) and, similarly to LPS (O-antigen) determine the serotype of microorganisms [4].

The capsule of *Azospirillum* has two high molecular weight polysaccharide-containing compounds termed lipopolysaccharide–protein complex (LPPC) and polysaccharide–lipid complex (PSLC) according to the ratio of the components [2]. These compounds contain carbohydrates, 3-hydroxylated fatty acids, and 2-keto-3-

Abbreviations: Ab, antibodies; COSY, correlation spectroscopy; CPS, capsular polysaccharides; DPS, *O*-deacetylated polysaccharide; EIA, enzyme immunoassay; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; KDO, 2-keto-3-deoxyoctonic acid; LPPC, lipopolysaccharide–protein complex; LPS, lipopolysaccharide (O-antigen); OPS, O-specific polysaccharide; PS, polysaccharide; PSLC, polysaccharide–lipid complex; ROESY, rotation nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

* To whom correspondence should be addressed.

deoxyoctonic acid (KDO), which are also specific for LPS [5]. Thus, LPPC and PSLC are supposed to be extracellular forms of LPS similarly to the first and fourth groups in the classification of capsular polysaccharides of *Escherichia coli* [6].

The earlier studies on the bacteria *A. brasilense* Sp7 and Sp245 with antibodies (Ab) to the glutaraldehyde-treated whole *Azospirillum* cells did not detect antigenic differences between LPS from capsular LPPC and O-polysaccharides from the outer membrane LPS [7]. Using polyclonal Ab to purified LPS specific for the polysaccharide moiety of O-antigen, it was shown that in the case of the *A. brasilense* strain Cd the capsule partially shielded LPS of the outer membrane [8]. Thus, the question of individual K-antigens in *Azospirillum* remained unsolved. Considering that CPS, due to their surface location, mediate the interaction of bacteria with environmental organisms and also data on their role in the interaction with plant and bacterial lectins [9, 10] and involvement in formation of biofilms [11] and other processes important for survival and functioning of microorganisms in association with plants [2, 4, 10], the absence of information about CPS structure is a significant gap in studies on molecular mechanisms of associativity.

Using rabbit Ab prepared by immunization with LPS and LPPC preparations, we have detected individual K-antigens in the typical strain *A. lipoferum* Sp59b and were the first to establish chemical structure of PS within CPS in bacteria of the *Azospirillum* genus.

MATERIALS AND METHODS

The typical strain *A. lipoferum* Sp59b (ATCC 29707, B-1519) from the All-Russian Collections of Microorganisms was used. The bacterium was cultured at 30°C on a liquid malate-saline medium supplemented with vitamins until the end of the exponential growth phase [5].

Isolation of polysaccharide-containing polymers from the surface of *Azospirillum*. The capsular matter was washed from the cell surface with 0.15 M NaCl during 6 days with daily change of the washing solution. LPS were isolated from the acetone-dried decapsulated cells with aqueous phenol by the Westphal method as described in [12]. The high molecular weight fraction of LPS 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). The column was eluted with 0.025 M NH_4HCO_3 (pH 8.3). The LPS yield was 2.6% of the dry cell weight.

The capsular matter washed within the two first days was concentrated, dialyzed for 24 h through membranes (exclusion limit of 12-14 kDa) against distilled water, and then subdivided into fractions of LPPC and PSLC by gel filtration on a column with Sepharose CL-4B. The LPPC were eluted with the column void volume, and then the PSLC fraction followed. The capsular complexes were separated several times to obtain maximally homogenous preparations (by gel-filtration data). The yields of LPPC and PSLC were, respectively, 0.3 and 0.1% of the dry bacterial cell weight.

The complexes were degraded with 2% acetic acid at 100°C for 5 h. The resulting precipitates were separated by centrifugation. The water-soluble fraction was separated by gel chromatography on a column with Sephadex G-50 in 0.05 M pyridine-acetate buffer (pH 4.5), monitoring the elution with a Knauer differential flow refractometer. As a result, polysaccharides were obtained with yields of 27.5 and 33.3% of the LPPC and PSLC weights, respectively.

LPS and CPS preparations were subjected to electrophoresis in 12.5% SDS-polyacrylamide gel [13]. The components were visualized by staining the gels with a silver nitrate-based dye [14] and with Coomassie Blue R-250 [15]. For immunoblotting, the separated components were transferred from polyacrylamide gel onto nitrocellulose membranes with pore diameter of 0.2 μm (Sigma, USA) in electric field at 60 V for 2 h. Free binding sites on the membrane were blocked with 1% defatted milk for 1 h. Immunodetection was performed by incubating the blots with polyclonal rabbit anti-LPS Ab [16] and LPPC from *A. lipoferum* Sp59b. The immunoblots were developed with horseradish peroxidase conjugated with goat anti-rabbit Ab (Sigma), 3,3′-diaminobenzidine, and hydrogen peroxide as substrate.

To prepare Ab against LPPC from *A. lipoferum* Sp59b ($Ab_{LPPC\ Sp59b}$), rabbits were immunized thrice with two-week intervals by subsequent injections of 0.5, 1.0, and 1.5 mg LPPC into popliteal lymphatic nodes. The LPPC solution was heated in advance at 100°C for 5 min and then centrifuged. For the first immunization the antigen was mixed at ratio 1 : 1 with complete Freund adjuvant and with incomplete Freund adjuvant at the subsequent immunizations. Blood was taken a week after the last immunization. Fractions of immunoglobulins G were prepared from the antisera by precipitation with ammonium sulfate [17].

The bacterial cells were agglutinated in 96-well plants as described in work [16]. Double radial immunodiffusion was performed routinely [18] in 1% agarose gel. The precipitate was stained with Coomassie Blue R-250. Solid-phase enzyme immunoassay (EIA) was performed in polystyrene 96-well plates (Medpolymer, Russia). The wells of the plates were supplemented with 50 μl of successive twofold dilutions of the specimens (in 0.15 M phosphate-saline buffer, pH 7.2). Goat anti-rabbit Ab conjugated with horseradish peroxidase (Sigma) were used as second antibodies. Hydrogen peroxide with *o*-phenylenediamine was used as substrate. Optical absorption of the samples was measured with an AIF-Ts-O1S immunoenzyme analyzer (ILIP, Russia) at λ = 490 nm.

The contents of carbohydrates, KDO, proteins, and nucleic acids in LPS, LPPC, and PSLC were determined colorimetrically by conventional methods described by us earlier in work [5]. The measurements were performed with a Specord 40 (Analytik Jena AG, Germany).

Analytical methods. Fatty acids in LPS were determined as methyl esters by GLC with a GL-2010 chromatograph (Shimadzu, Japan). The acids were methylated as described in [19]. Monosaccharides were analyzed and absolute configurations of neutral sugars as polyol acetates and acetylated glycosides, respectively, were established by GLC using optically active alcohol (R)-2-octanol. Specimens were prepared as described earlier [20].

Polysaccharides (20 mg) were *O*-deacetylated by treatment with 13% ammonium hydroxide at 37°C for 16 h, and the resulting deacetylated polysaccharide (DPS ~ 18 mg) was desalted by chromatography on a TSK HW-40 gel (Toyo Soda, Japan) in water.

NMR-spectroscopy of OPS. NMR spectra were obtained with a DRX-500 spectrometer (Bruker, Germany) in solution of 99.96% D_2O at 27°C (with acetone as internal standard, $\delta_H = 2.225$, $\delta_C = 31.45$). The specimens were previously lyophilized twice 99.9% D_2O. Two-dimensional spectra were obtained using the standard software from Bruker; the data were collected and treated using the XWINNMR 2.1 program. In experiments with TOCSY and NOESY the mixing time was 150 and 200 msec, respectively.

Results of all experiments were processed statistically.

RESULTS AND DISCUSSION

Surface glycopolymers LPPC, PSLC, and LPS were obtained by gel filtration on Sepharose CL-4B from the capsular material and aqueous–phenolic extract of acetone-dried bacterial *A. lipoferum* Sp59b cells. These preparations contained approximately the same amount of carbohydrates (~40%) and different quantities of protein (0.2% in LPS, ~7 and 3% in LPPC and PSLC, respectively). In all glycopolymers KDO was identified: 4.5% in LPS, 2.8% in LPPC, and 0.2% in PSLC. The preparations also contained lipid components (the lipid precipitate after acidic degradation of the complexes was 20-30% of the initial preparation weight), core components, and polynucleotides as admixtures.

KDO is known to be an obligatory component of LPS. However, KDO was also found in CPS, in particular, in some strains of *E. coli* [6, 21], *Actinobacillus pleuropneumoniae* [22-24], *Neisseria meningitidis* [25], and *Azospirillum* [5].

Differences were revealed in the structure and ratio of fatty acids in the lipid components of the preparations. Thus, in the lipid A from LPS $C_{12:0}$, 3-OH-$C_{12:0}$, 2-OH-$C_{12:0}$, 3-OH-$C_{14:0}$, $C_{16:0}$, and $C_{18:1}$ acids were prevalent, and their content was 21.9, 30.7, 8.2, 12.6, 13.0, and 10.1%, respectively, of all identified methyl esters of the fatty acids. In LPPC and PSLC 3-OH-$C_{14:0}$ was prevalent (33.2 and 19.1%, respectively), the $C_{18:1}$ content was ~30% in both preparations, the content of 3-OH-$C_{16:0}$ was 14.2 and 9.4%, respectively, and the content of $C_{16:0}$ was 6.9 and 8.2%, respectively. In PSLC the contents of $C_{16:0}$ and $C_{19:0}$ were, respectively, ~3 and ~5%, and in LPPC $C_{12:0}$ (~6%) was also identified. Because the lipid component determines endotoxic properties of LPS, it was suggested that differences in the structure of lipid acids in LPPC, PSLC, and LPS of *Azospirillum* should markedly influence the sub-molecular structure of these glycopolymers and their biological activity. Note that 3-hydroxyalcanic acids are obligatory and, as a rule, prevalent components of the lipid A of LPS (the share of these acids usually is 50-75% of the total acids) [1]. Determination of the profile of saturated hydroxyacids is often used for detection of endotoxins in various biological samples [26] and soil [27], and also as an additional chemotaxonomic character for elucidation of phylogenetic relations between microorganisms [28]. Octadecenoic acid is characteristic for the *Proteobacteria* α-subclass (its share is usually 40-70% of the total content of fatty acids in the cells) [29]. This acid was also identified in the lipid A of LPS from the bacterium *A. lipoferum* SpBr17 [30].

SDS-PAGE with subsequent visualization of glycopolymers with a silver nitrate-based dye (Fig. 1a) revealed a significant difference in the migration rate of molecules of capsular complexes and LPS. All polysaccharide-containing polymers were heterogenous in molecular weight. Note that high molecular weight fractions were prevalent in LPPC and PSLC, whereas LPS were characterized by presence of both S- and R-form molecules. The staining of the preparations after separation in SDS-PAGE by Coomassie Blue R-250 revealed the absence of protein in LPS, whereas LPPC and PSLC had seven subunits with apparent molecular weight of 19-45 kDa with a dominating 40-kDa monomer (Fig. 1e). Electrophoresis of outer membrane proteins from the porin group revealed in enterobacteria (*Pseudomonas*, *Yersinia*) and also in bacteria of the *Azospirillum* genus an oligomeric protein as a multiplet band in the region of molecular weights of 90-120 kDa. Heating to 100°C and treatment with a detergent converted the protein into monomers with apparent molecular weight of 14-20 and 40 kDa [31, 32]. In the *A. brasilense* strains Sp245 and S17, electrophoretic mobilities of LPPC proteins from the capsule and the outer membrane were the same under denaturing conditions (~40 kDa) [31]. The same was also found by us for the *A. lipoferum* strain Sp59b.

To detect antigenic properties of polysaccharides from the capsular matter of *Azospirillum*, polyclonal rabbit antibodies were obtained to LPPC from *A. lipoferum* Sp59b ($Ab_{LPPC\ Sp59b}$). The protein component was inacti-

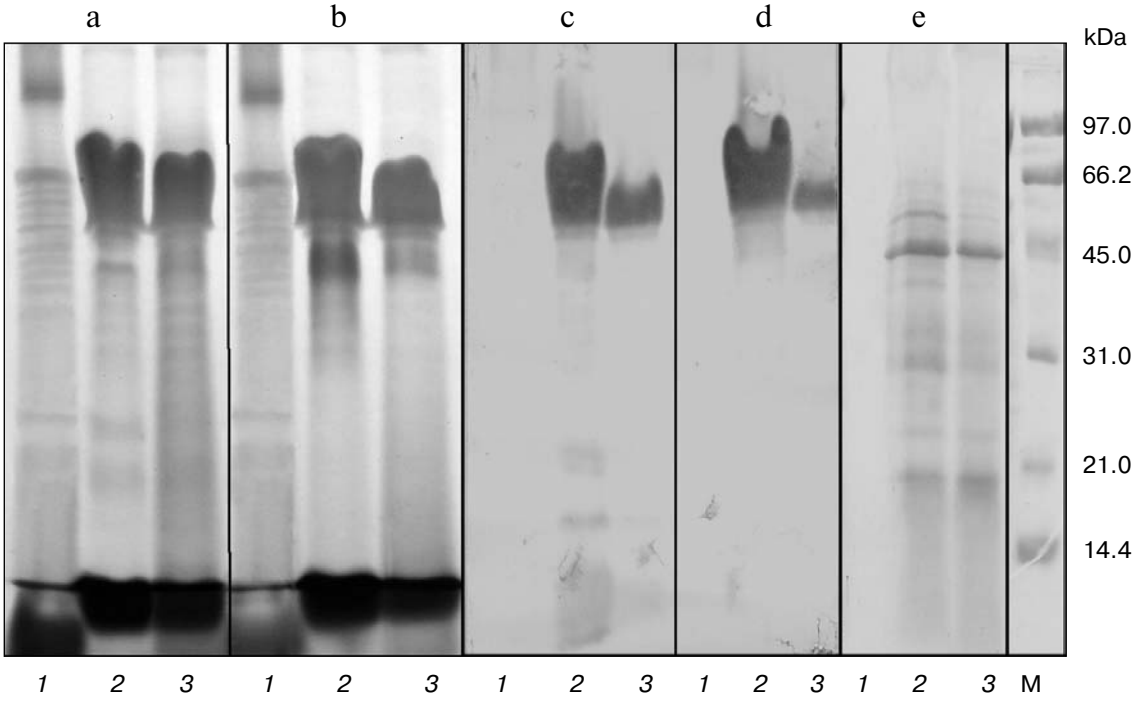


Fig. 1. SDS-PAGE (staining with silver nitrate upon oxidation with periodate (a, b) and staining with Coomassie Blue R-250 (e)) and immunoblotting with rabbit $Ab_{LPPC\ Sp59b}$ (c, d) of LPS (*1*), LPPC (*2*), and PSLC (*3*) from *A. lipoferum* Sp59b. M, protein markers. Preparations were treated with proteinase K (b, d).

vated by boiling the preparation before the immunization. The antiserum titer was 1 : 40,960, two orders higher than the activity of the antiserum prepared upon immunization of laboratory animals with LPS of the same strain. This was due to the high antigenic activity of extracellular toxins (similar to the whole microbial cell activity) even with very low content (traces) of the protein component [3]. The high specific antigenicity and serological activity suggest the protective function of these polymers. The activities of $Ab_{LPPC\ Sp59b}$ and antibodies to LPS ($Ab_{LPS\ Sp59b}$) prepared earlier [16] were compared respectively to the studied bioglycans and the *A. lipoferum* Sp59b cells. The capsular material was washed daily from the bacterial cells during 6 days, changing the washing solution every day. It was shown by solid phase EIA and double radial immunodiffusion that none of the collected fractions of the capsular complexes from *A. lipoferum* Sp59b interacted with $Ab_{LPS\ Sp59b}$ (Fig. 2).

$Ab_{LPS\ Sp59b}$ did not interact with homologous native bacterial cells but induced agglutination of cells with removed capsule. The procedure for preparing viable decapsulated cells of *Azospirillum* was developed by us earlier and described in detail in work [12]. The minimal concentration of IgG inducing agglutination of the decapsulated cells was 0.2 mg/ml.

LPPC, PSLC, and LPS from *A. lipoferum* Sp59b were tested by immunoblotting for the presence of common antigenic determinants (data not presented), and $Ab_{LPS\ Sp59b}$ were shown to interact with LPS but not with LPPC and PSLC. On similar analysis of $Ab_{LPPC\ Sp59b}$, LPS and capsular complexes were separated by electrophoresis in two variants: with preliminary treatment of specimens with proteinase K and without it. The proteolysis was performed to prevent distortion of results from the interaction of protein components of the capsular complexes with Ab. The treatment with the enzyme resulted in disappearance of bands in the lower parts of the lanes in the electrophoretic profile of the capsular glycopolymers (Fig. 1b, lanes *2* and *3*). The immunoblotting data indicated that $Ab_{LPPC\ Sp59b}$ did not interact with LPS, but it interacted with LPPC and PSLC (Fig. 1, c and d). In the general pool of $Ab_{LPPC\ Sp59b}$, Ab specific to its carbohydrate component were prevalent (Fig. 1 (c and d), lanes *2* and *3*) and only a small portion was represented by Ab to protein (Fig. 1c, lane *2*). Data of this experiment also indicated that $Ab_{LPPC\ Sp59b}$ reveals common antigenic determinants in both LPPC and PSLC (Fig. 1, c and d). Thus, immunochemical studies revealed the difference in the structure of antigenic determinants of LPS of the outer membrane and CPS of *A. lipoferum* Sp59b.

Polysaccharides of capsular complexes were prepared by mild acidic degradation of LPPC and PSLC with subsequent chromatography of the water-soluble moiety on Sephadex G-50. Studies by GLC of polyol acetates and (R)-2-octylglycosides resulting from the complete hydrolysis of polysaccharides indicated that

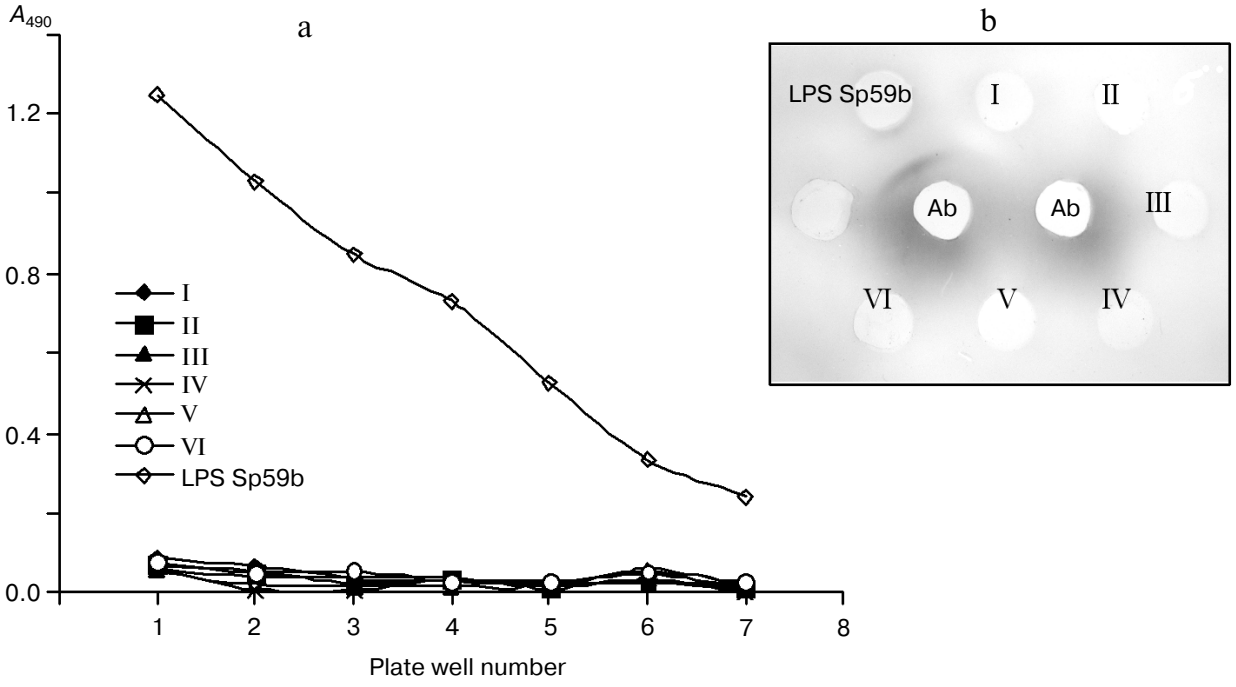


Fig. 2. Enzyme immunoassay (a) and immunodiffusion analysis (b) of the capsular matter and LPS from *A. lipoferum* Sp59b with $Ab_{LPS\ Sp59b}$. I-VI, fractions of capsular matter obtained on the corresponding day of cell washing free of the capsule.

they had the identical monosaccharide composition and consisted of L-Rha and D-Glc in the ratio of ~2.7 : 1.

Methylation with the subsequent GLC-MS of partially methylated polyol 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. Based on these data, the repeating units of PS isolated from LPPC and PSLC were concluded to

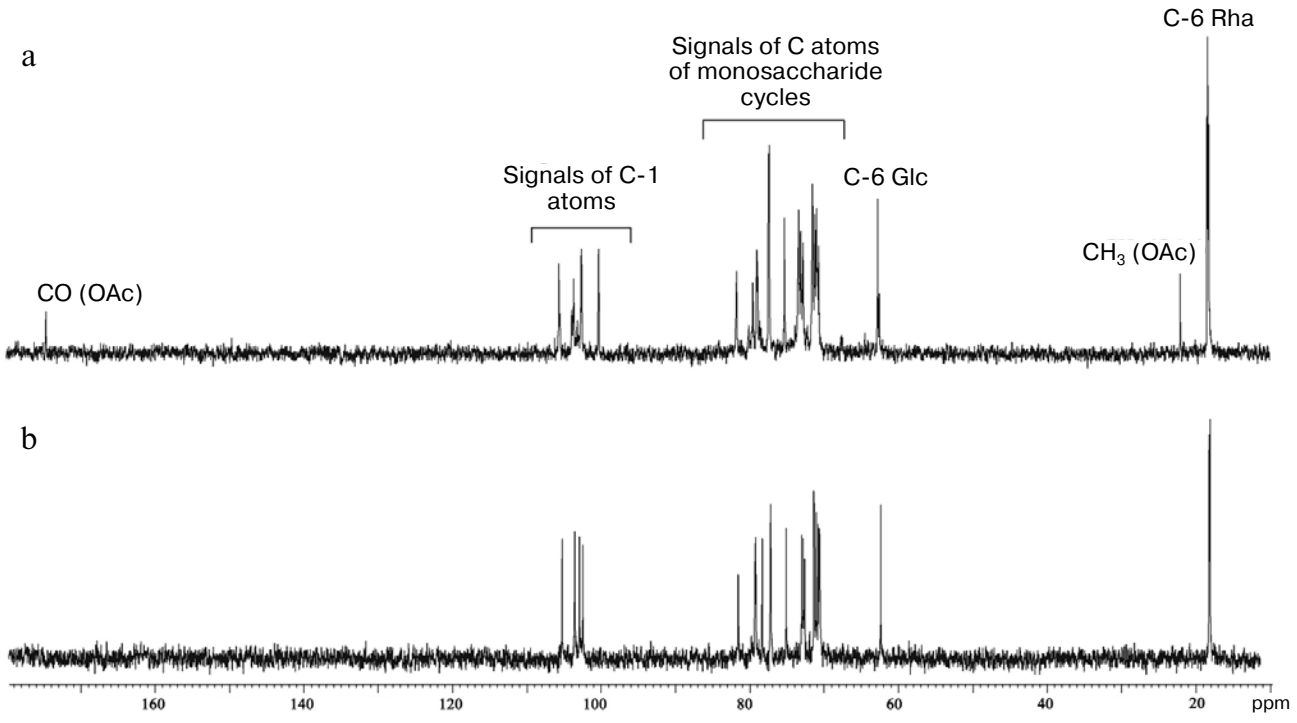


Fig. 3. ^{13}C-NMR spectrum of intact (a) and *O*-deacetylated (b) polysaccharide isolated from LPPC of *A. lipoferum* Sp59b.

Data of 500-MHz ^{1}H-NMR and 125-MHz ^{13}C-NMR spectra of *O*-deacetylated polysaccharide (chemical shifts, ppm)

Monosaccharide residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
→2,3)-α-L-Rha*p*^{I}-(1→	5.15 102.5	4.36 78.3	4.08 81.6	3.65 72.7	3.89 70.6	1.33 18.2	–
→3)-α-L-Rha*p*^{II}-(1→	5.04 103.5	4.14 71.4	3.93 79.3	3.58 73.0	3.86 70.6	1.28 18.1	–
→3)-α-L-Rha*p*^{III}-(1→	5.15 102.9	4.14 71.3	3.86 79.2	3.54 72.9	3.76 70.8	1.27 18.0	–
β-D-Glc*p*-(1→	4.67 105.2	3.35 75.1	3.44 77.2	3.41 71.1	3.49 77.2	3.94 62.4	3.77

consist of residues of 3-substituted Rha, 2,3-disubstituted Rha in the branching point, and the terminal residue Glc.

Note that the ^{13}C- and ^{1}H-NMR spectra of polysaccharides were identical; therefore, we describe the NMR spectra only of PS from LPPC. The ^{13}C-NMR spectrum of the polysaccharide contained signals of different intensity (Fig. 3a), possibly because of non-stoichiometric *O*-acetylation, which was confirmed by the CH_3 signal of the *O*-acetyl group at 21.8 ppm. Therefore, the PS was *O*-deacetylated by treatment with NH_4OH and the resulting *O*-deacetylated polysaccharide (DPS) was studied using NMR spectroscopy approaches.

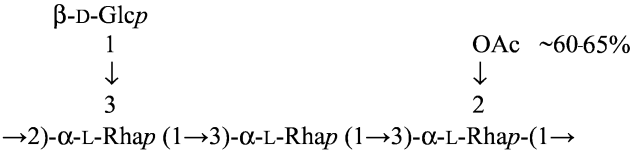
In the ^{13}C-NMR spectrum (Fig. 3b) there were signals of four anomeric carbon atoms at 102.5-105.2 ppm, 16 carbon atoms of monosaccharide cycles in the region of 62.4-81.6 ppm, and also three methyl groups (C-6 Rha at 18 ppm). The absence of signals in the region of 82-88 ppm characteristic for furanosides indicated that all monosaccharide residues were present as pyranosides. Respectively, in the ^{1}H-NMR spectrum of DPS there were signals of four anomeric protons at 4.67-5.15 ppm and also signals of a group of protons of monosaccharide residues at 3.35-4.49 ppm and three CH_3-groups of rhamnoses at 1.27-1.33 ppm. Thus, DPS consists of tetrasaccharide repeating units, each of which includes three Rha and one Glc residues.

The ^{1}H- and ^{13}C-NMR spectra of DPS were assigned using two-dimensional experiments COSY, TOCSY, ROESY, ^{1}H/^{13}C HSQC, and HMBC (table). The two-dimensional ROESY experiment indicated the presence of cross-peaks between anomeric protons and protons at the binding carbon atoms at 4.67/4.08, 5.15/4.36, 5.15/3.93, and 5.04/3.86, which were assigned to inter-unit correlations H-1 Glc/H3 Rha^{I}, H-1 Rha^{III}/H-2 Rha^{I}, H-1 Rha^{I}/H-3 Rha^{II}, and H-1 Rha^{II}/H-3 Rha^{III}, respectively. These data corresponding to locations of monosaccharide substituents established by chemical shifts using ^{13}C-NMR revealed the monosaccharide sequence in DPS.

A relatively high value of spin–spin coupling constant ($J_{1,2}$ ~ 7 Hz) indicated that the Glc residue was attached through a β-glycoside bond. The location H-1,3,5 of signals of all rhamnose residues (Rha^{I}-Rha^{III}) in the relatively low field at 5.04-5.15, 3.86-4.08, and 3.76-3.89 ppm, respectively, and also location of C-5 signals at 70.6-70.8 ppm suggested that these residues should be α-bound (according to data published for β-Rha*p* and α-Rha*p*) [33, 34].

The location of the *O*-acetyl group on the initial PS was established by ROESY and HMBC experiments. Considering a weak-field shift (5.35 ppm) of proton H-2 of the Rha^{III} residue and a 73.2-ppm chemical shift of C-2 of the same residue, it was suggested that Rha^{III} should be partially *O*-acetylated in position 2. A correlation was also observed between H-2 of Rha^{III} and protons of the CH_3-group and the carboxyl group carbon atom. The acetylation degree of Rha^{III} residues was determined to be 60-65% by analyzing integral intensities of the proton spectrum. Note that the presence of OAc-group noticeably changed the positions of ^{1}H- and ^{13}C-NMR signals of *O*-acetylated Rha^{III} residue and slightly changed those of Rha^{I} (Fig. 4).

Based on the findings, the repeating units of PS from LPPC and PSLC of *A. lipoferum* Sp59b are concluded to be identical tetrasaccharides with the following structure:



Note that the structure of this PS is very similar to structures of OPS of the bacteria *A. lipoferum* SR65 and SpBr17 [35, 36]. They are different by the absence of acetylation of OPS of the *A. lipoferum* strain SR65, whereas OPS of *A. lipoferum* SpBr17 is 100% acetylated. However, the repeating unit of OPS of *A. lipoferum* Sp59b is a branched hexasaccharide [37]:

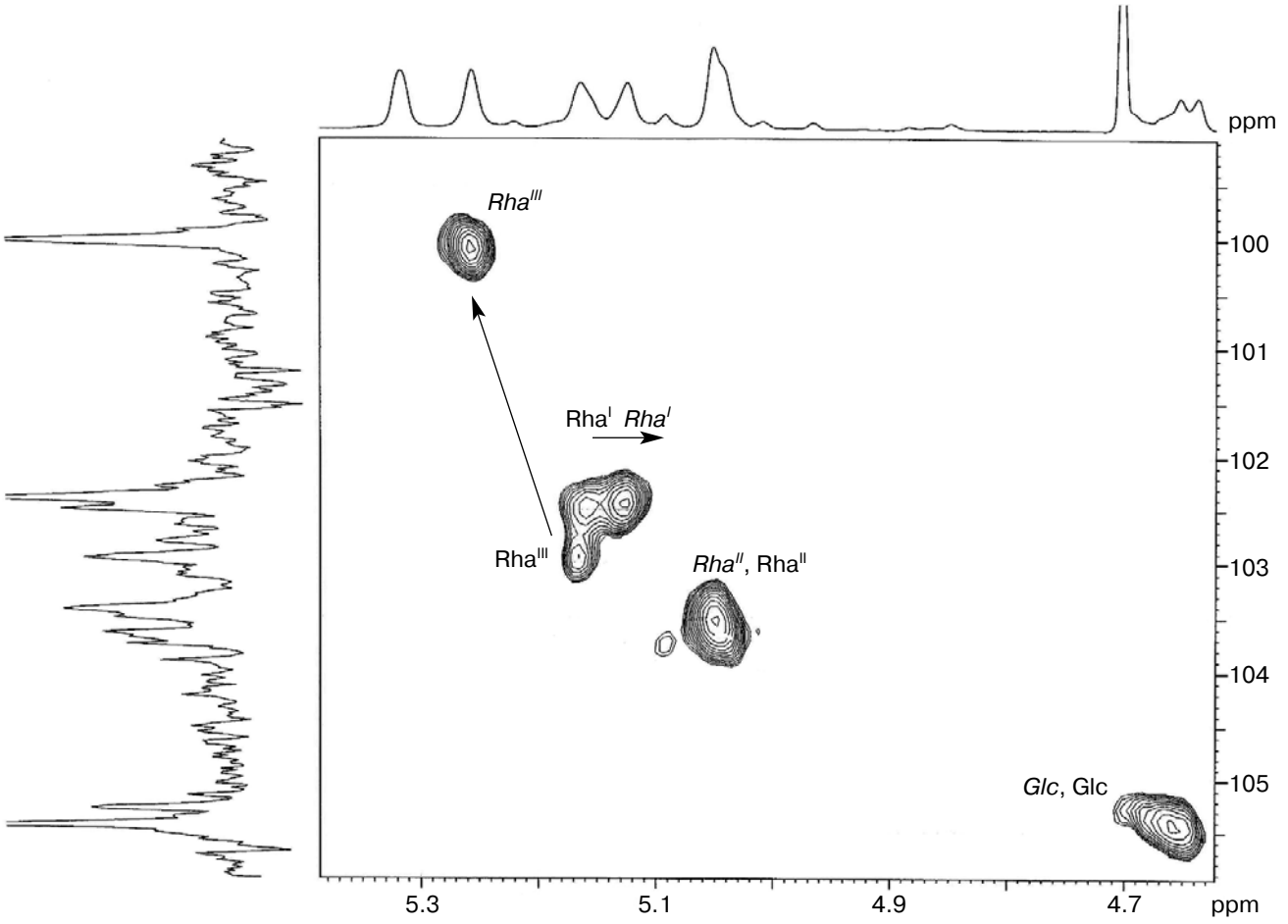


Fig. 4. Fragment of anomeric region of two-dimensional ^{1}H- and ^{13}C-HSQC spectrum of polysaccharide isolated from LPPC of *A. lipoferum* Sp59b. Anomeric signals in *O*-acetylated repeating units are printed in italics and *O*-deacetylated units in roman. The arrows show the signal shift induced by *O*-acetylation.

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→

It seems very interesting that all mentioned polysaccharides of *Azospirillum* contain the structural fragment [α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)], which is widely distributed in OPS of different phytopathogenic bacteria. Production of this fragment in OPS of *Rhizobium* sp. NGR234 under the influence of plant flavonoids is necessary for nodulation of Leguminosae and is a part of the symbiotic interaction [38].

Comparison of structures of repeating units of polysaccharides from LPS, LPPC, and PSLC of *A. lipoferum* Sp59b and also of profiles of fatty acids of their lipid moieties shows that this strain grown on liquid medium with sodium malate as a source of carbon synthesizes individual K-antigens. Structural differences of CPS and LPS are also described for other microorganisms, e.g. for some strains of *Vibrio cholerae* [39], *Actinobacillus pleuropneumoniae* [40], and *Burkholderia* (*Pseudomonas*) *pseudomallei* [41]. Possibly, the differences found in the structure of capsular and membrane polysaccharides are associated with the involvement of these components in different stages of formation of the plant–bacterial association (by analogy with known data on the role of surface glycopolymers of rhizobia in formation of the symbiosis with Leguminosae).

This work was supported by the Russian Foundation for Basic Research (project No. 08-04-00669) and the Grant Counsel of the Russian Federation President for supporting leading scientific schools (NSh 3171.2008.4).

REFERENCES

1. Michiels, K. W., Crors, C. L., and Vanderleyden, J. (1991) *J. Gen. Microbiol.*, **137**, 2241-2246.

2. Skvortsov, I. M., and Ignatov, V. V. (1998) *FEMS Microbiol. Lett.*, **165**, 223-229.
3. Zdorovenko, G. M. (1988) *Mikrobiol. Zh.*, **50**, 98-107.
4. Ovodov, Yu. S. (2006) *Biochemistry (Moscow)*, **71**, 937-954.
5. Konnova, S. A., Makarov, O. E., Skvortsov, I. M., and Ignatov, V. V. (1994) *FEMS Microbiol. Lett.*, **118**, 93-99.
6. Whitfeld, C., and Roberts, I. S. (1999) *Mol. Microbiol.*, **31**, 1307-1319.
7. Matora, L. Yu., and Shchegolev, S. Yu. (2002) *Mikrobiologiya*, **71**, 211-214.
8. Konnova, O. N., Burygin, G. L., Fedonenko, Yu. P., Matora, L. Yu., Pankin, K. E., Konnova, S. A., and Ignatov, V. V. (2006) *Mikrobiologiya*, **75**, 383-388.
9. Del Gallo, M., Negi, M., and Neyra, C. A. (1989) *J. Bacteriol.*, **171**, 3504-3510.
10. Yegorenkova, I. V., Konnova, S. A., Sachuk, V. N., and Ignatov, V. V. (2001) *Plant Soil*, **231**, 275-282.
11. Lasa, I. (2006) *Int. Microbiol.*, **9**, 21-28.
12. Fedonenko, Yu. P., Egorenkova, I. V., Konnova, S. A., and Ignatov, V. V. (2001) *Mikrobiologiya*, **70**, 384-390.
13. Hitchcock, P. J., and Brown, T. M. (1983) *J. Bacteriol.*, **154**, 269-277.
14. Tsai, C. M., and Frasch, C. E. (1982) *Analyt. Biochem.*, **119**, 115-119.
15. Fazekas, St. G., Webster, R. G., and Datyner, A. (1963) *Biochim. Byophys. Acta*, **71**, 377.
16. Konnova, O. N., Boiko, A. S., Burygin, G. L., Fedonenko, Yu. P., Matora, L. Yu., Konnova, S. A., and Ignatov, V. V. (2008) *Mikrobiologiya*, **77**, 383-388.
17. Cabot, E., and Mayer, M. (1968) in *Experimental Immunology* [Russian translation], Meditsina, Moscow.
18. Ouchterlony, O., and Nilsson, L.-A. (1979) *Handbook Experimental Immunology* (Weiz, D. M., ed.) Vol. 1, Alden Press, Oxford, pp. 19-33.
19. Mayer, H., Tharanathan, R. N., and Weckesser, J. (1985) *Meth. Microbiol.*, **18**, 157-207.
20. Fedonenko, Yu. P., Konnova, O. N., Zatonsky, G. V., Shashkov, A. S., Konnova, S. A., Zdorovenko, E. L., Ignatov, V. V., and Knirel, Yu. A. (2004) *Carbohydr. Res.*, **339**, 1813-1816.
21. Jann, B., and Jann, K. (1990) *Curr. Top. Microbiol. Immunol.*, **150**, 19-42.
22. Altman, E., Brisson, J. R., and Perry, M. B. (1987) *Eur. J. Biochem.*, **170**, 185-192.
23. Beynon, L. M., Richards, J. C., and Perry, M. B. (1991) *Carbohydr. Res.*, **220**, 185-193.
24. Altman, E., Brisson, J. R., Gagne, S. M., and Perry, M. B. (1992) *Eur. J. Biochem.*, **204**, 225-230.
25. Bhattacharjee, A. K., Jennings, H. J., and Kenney, C. P. (1978) *Biochemistry*, **17**, 645-651.
26. Maitra, S. K., Nachum, R., and Pearson, F. C. (1986) *Appl. Environ. Microbiol.*, **52**, 510-514.
27. Parker, J. H., Smith, G. A., Fredrickson, H. L., Vestal, J. R., and White, D. C. (1982) *Appl. Environ. Microbiol.*, **44**, 1170-1177.
28. Heike, R. U. S., Freudenberg, M., Weckesser, J., and Mayer, H. (1995) *Arch. Microbiol.*, **164**, 280-289.
29. Busse, H. J., Denner, E. B. M., and Lubitz, W. (1996) *J. Biotech.*, **47**, 3-38.
30. Choma, A., and Komaniecka, I. (2008) *Carbohydr. Res.*, **343**, 799-804.
31. Konnova, S. A., and Ignatov, V. V. (2003) *Biology of Plant–Microbe Interaction (Proc. 11th Int. Congr. on Molecular Plant–Microbe Interaction)*, Vol. 4 (Tikhonovich, I., Lugtenberg, B., and Provorov, N., eds.) St.-Petersburg, pp. 327-331.
32. Vostrikova, O. P., Kim, N. Yu., Likhatskaya, G. N., Guzev, K. V., Vakorina, T. I., Khomenko, V. A., Novikova, O. D., and Soloveva, T. F. (2006) *Bioorg. Khim.*, **32**, 371-383.
33. Jansson, P.-E., Kenne, L., and Widmalm, G. (1989) *Carbohydr. Res.*, **188**, 169-191.
34. Lipkind, G. M., Shashkov, A. S., Knirel, Y. A., Vinogradov, E. V., and Kochetkov, N. K. (1988) *Carbohydr. Res.*, **175**, 59-75.
35. Fedonenko, Yu. P., Zdorovenko, E. L., Konnova, S. A., Kachala, V. V., and Ignatov, V. V. (2008) *Carbohydr. Res.*, **343**, 2841-2844.
36. Choma, A., Komaniecka, I., and Sowinski, P. (2009) *Carbohydr. Res.*, **344**, 936-939.
37. Fedonenko, Yu. P., Konnova, O. N., Zatonsky, G. V., Konnova, S. A., Kocharova, N. A., Zdorovenko, E. L., and Ignatov, V. V. (2005) *Carbohydr. Res.*, **340**, 1259-1263.
38. Newman, M.-N., Dow, J. M., Molinaro, A., and Parrilli, M. (2007) *J. Endotoxin Res.*, **13**, 69-84.
39. Chen, Y., Bystricky, P., Adeyeye, J., Panigrahi, P., Ali, A., Johnson, J. A., Bush, C. A., Morris, J. G., and Stine, O. C. (2007) *BMC Microbiol.*, **20**, 1-15.
40. MacLean, L. L., Perry, M. B., and Vinogradov, E. (2004) *Infect. Immun.*, **72**, 5925-5930.
41. Masoud, H., Ho, M., Schollaardt, T., and Perry, M. B. (1997) *J. Bacteriol.*, **179**, 5663-5669.