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Note

Structural analysis of the O-antigen of the lipopolysaccharide from *Azospirillum lipoferum* SR65

Yuliya P. Fedonenko [a], Evelina L. Zdorovenko [b,*], Svetlana A. Konnova [a,c], Vadim V. Kachala [b], Vladimir V. Ignatov [a]

[a] *Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Prospekt Entuziastov 13, 410049 Saratov, Russia*
[b] *N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, 119991 Moscow, Russia*
[c] *Saratov State University, Astrakhanskaya Street 83, 410012 Saratov, Russia*

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ABSTRACT

A neutral O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated by phenol/water extraction from the asymbiotic diazotrophic rhizobacterium *Azospirillum lipoferum* SR65. The following structure of the O-polysaccharide was established by composition and methylation analyses, Smith degradation, and ^{1}H and ^{13}C NMR spectroscopy, including a 2D ROESY experiment:

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

Nitrogen-fixing Gram-negative soil bacteria of the genus *Azospirillum* have been used as models to study associative plant–microbial relations.[1,2] The ability of the azospirilla to exert a positive effect on the growth and productivity of a wide range of plants attests that these bacteria have a high adaptation potential and are promising for application as biofertilizers. Surface polysaccharides have been known to be important both for survival of *Azospirillum* in adverse environments and for its interaction with the roots of plants.[3–5] Data indicate that the lipopolysaccharides (LPSs) of the *Azospirillum* outer membrane are involved in the formation of bacterial associations with the roots of bread cereals. In particular, mutants defective in LPS synthesis are worse adsorbers to wheat roots[6] and worse colonizers of maize roots[7] than are their nondefective counterparts. Moreover, the addition of wheat-root exudates to an *Azospirillum* growth medium changes the LPS electrophoretic profile.[8] Although bacterial-surface glycopolymers have long been recognized as important for the establishment of plant–microbe associations, they remain underinvestigated. Particularly, this is true of the LPS. In previous work, we compared the O-polysaccharides structures of the LPSs of azospirillar strains isolated from the roots and rhizospheres of various plants in various regions of the world, including *Azospirillum brasilense* Sp245, SR75, and Cd; *Azospirillum irakense* KBC1; and *Azospirillum lipoferum* Sp59b.[9–13] Now we report the structure for the O-polysaccharide of *A. lipoferum* SR65. This strain was isolated from the rhizosphere of wheat grown on poor saline soil in a Saratov region arid zone with a sharply continental climate. Strain SR65 is of interest as a promising inoculant for bread cereals grown in extreme environments.

Bacterial cells were extracted with aq 45% phenol, and the LPS was recovered from the aqueous phase. Degradation of the LPS under mild acid conditions afforded a lipid sediment and a water-soluble carbohydrate portion, which was fractionated by GPC on Sephadex G-50 to give a O-specific polysaccharide (OPS). Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the OPS revealed rhamnose and glucose in 2.1:1 ratios. Determination of the absolute configurations of the monosaccharides by GLC of the acetylated glycosides with (*S*)-2-octanol indicated that Rha is L and Glc is D.

Methylation analysis of the OPS by GLC–MS of the partially methylated alditol acetates resulted in identification of

* Corresponding author. Tel.: +7 495 1376148; fax: +7 495 1355328.
E-mail address: evelina@ioc.ac.ru (E. L. Zdorovenko).

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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. Therefore, the OPS is branched with lateral glucose residues and 2,3-substituted rhamnose residues at the branching point.

Each of the ^{1}H and ^{13}C NMR spectra of the OPS (Figs. 1 and 2) contained signals for four monosaccharide residues. The spectra were assigned using ^{1}H,^{1}H COSY, TOCSY, ROESY, and H-detected ^{1}H,^{13}C HSQC experiments (Tables 1 and 2). The sugar spin systems were identified by tracing connectivities in the TOCSY spectrum starting from H-1 of Glc*p* and from either H-1 or H-6 of Rha*p* residues combined with the characteristic coupling pattern and $^{3}J_{H,H}$ coupling constant values.

The assignment for Glc*p* was confirmed by an H-1,H-5 correlation observed in the ROESY spectrum, which, together with a relatively large $J_{1,2}$ coupling constant of ~8 Hz, indicated the β-linkage of this residue. The α-linkage of all three rhamnose residues (Rha*p*^{I}-Rha*p*^{III}) was determined by comparison of the C-5 chemical shifts (δ 68.7–70.8) with those of the corresponding α- and β-rhamnopyranosides.[14]

The ^{13}C NMR chemical shift data (Table 2 and Fig. 1) revealed low-field displacements of the signals for Rha*p*^{I} C-2 and C-3, Rha*p*^{II} C-3, and Rha*p*^{III} C-3, as compared with their positions in the corresponding non-substituted monosaccharides.[10] These displacements were due to glycosylation and defined the linkage pattern in the repeating unit. In agreement with the terminal position of

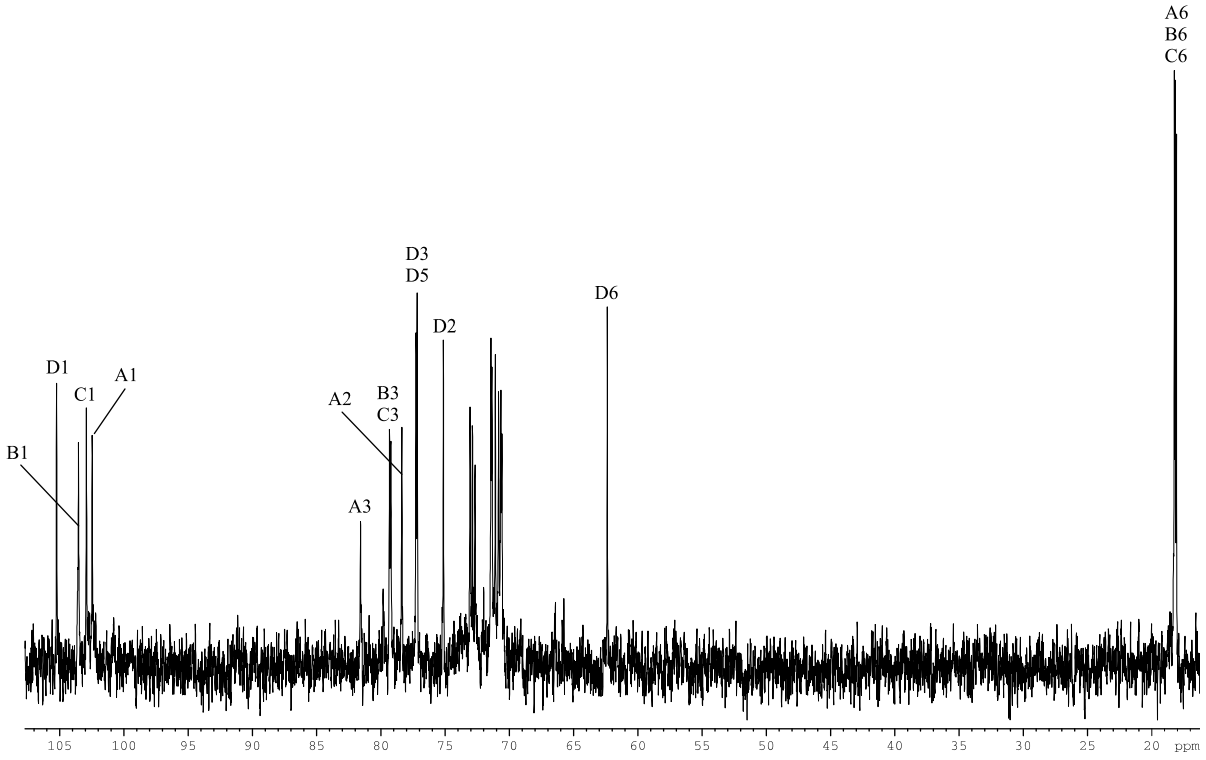

Figure 1. ^{13}C NMR spectrum of the polysaccharide from *A. lipoferum* SR65.

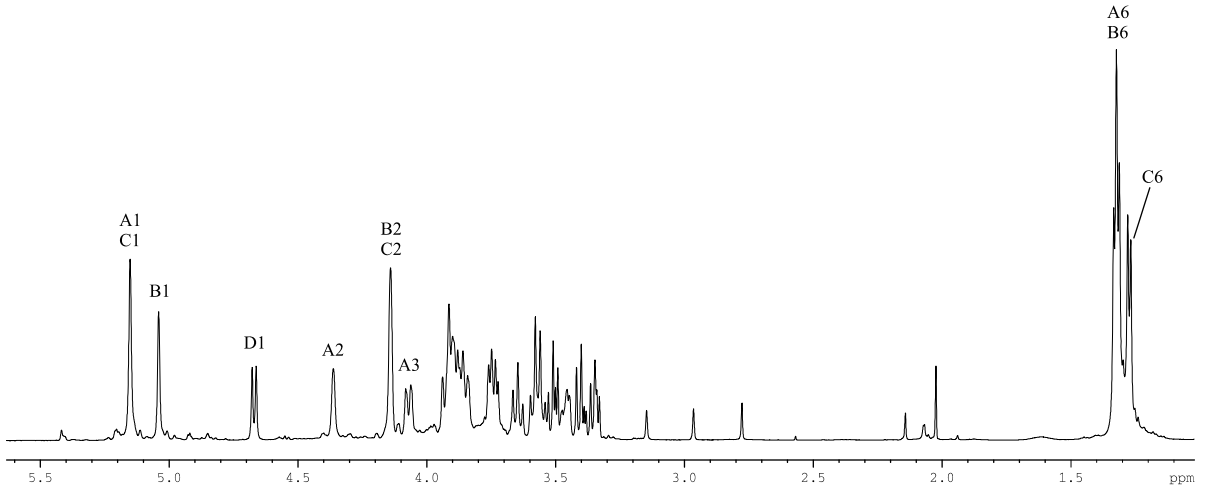

Figure 2. ^{1}H NMR spectrum of the polysaccharide from *A. lipoferum* SR65.

Table 1
500-MHz ^{1}H NMR data (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
PS							
→2,3)-α-L-Rha*p*^{I}-(1→(A)	5.15	4.36	4.08	3.65	3.89	1.32	—
→3)-α-L-Rha^{II}*p*-(1→(B)	5.04	4.14	3.93	3.58	3.86	1.32	—
→3)-α-L-Rha^{III}*p*-(1→(C)	5.15	4.14	3.86	3.54	3.76	1.27	—
β-D-Glc*p*-(1→(D)	4.67	3.35	3.44	3.41	3.49	3.94	3.77
Smith-degraded PS							
→2)-α-L-Rha*p*^{I}-(1→	5.22	4.09	3.97	3.52	3.78	1.33	
→3)-α-L-Rha^{II}*p*-(1→	4.97	4.18	3.86	3.58	3.89	1.33	
→3)-α-L-Rha^{III}*p*-(1→	5.04	4.15	3.93	3.58	3.87	1.30	

Table 2
125-MHz ^{13}C NMR data (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
PS						
→2,3)-α-L-Rha*p*^{I}-(1→(A)	102.5	78.3	81.6	72.7	70.6	18.2
→3)-α-L-Rha*p*^{II}-(1→(B)	103.5	71.4	79.3	73.0	70.6	18.1
→3)-α-L-Rha*p*^{III}-(1→(C)	102.9	71.3	79.2	72.9	70.8	18.0
β-D-Glc*p*-(1→ (D)	105.2	75.1	77.2	71.1	77.2	62.4
Smith-degraded PS						
→2)-α-L-Rha*p*^{I}-(1→	102.4	79.6	71.4	73.8	70.4	18.5
→3)-α-L-Rha^{II}*p*-(1→	103.8	71.2	79.5	72.9	70.4	18.5
→3)-α-L-Rha^{III}*p*-(1→	104.0	71.5	79.3	72.9	70.6	18.5

Glc*p*, the C-2–C-6 chemical shifts of this residue were close to the values for unsubstituted α-glucopyranose.[14] The monosaccharide sequence in the repeating unit was determined by the following correlations between anomeric protons and protons at the linkage carbons in the ROESY spectrum: Rha*p*^{I} H-1, Rha*p*^{II} H-3; Rha*p*^{II} H-1,Rha*p*^{III} H-3; Rha*p*^{III} H-1,Rha*p*^{I} H-2; and Glc*p*H-1, Rha*p*^{I} H-3.

The OPS structure was confirmed by Smith degradation, which resulted in oxidation of the terminal glucose in the side chain to give a modified polysaccharide (DPS). Its ^{1}H and ^{13}C NMR spectra showed three signals in the region of anomeric atoms at δ_H 4.97–5.22 and δ_C 102.4–103.5. The structure of the DPS as a linear α-1→3/α-1→2-linked rhamnan was established by 2D ^{1}H and ^{13}C NMR spectroscopy, as described above for the OPS (assigned ^{1}H and ^{13}C NMR chemical shifts are tabulated in Tables 1 and 2).

Therefore, the O-polysaccharide of the LPS recovered from the aqueous layer after phenol–water extraction of *A. lipoferum* SR65 is neutral and has the branched tetrasaccharide repeating unit shown in Chart 1 (structure **1**).

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

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

β-D-Glc*p*
1 OAc
↓ |
2 2
→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ **3**

Chart 1. Structures of the O-polysaccharide repeating units of *A. lipoferum* SR65 (**1**), *A. brasilense* S17 (**2**), and *A. lipoferum* SpBr17 (**3**).

The established structure further extends the list of *Azospirillum* O-polysaccharides that are enriched with D- or L-rhamnose entering into both main and side chains. Interestingly, the O-polysaccharides of *A. lipoferum* SR65 studied in this work and *A. brasilense* S17 (OPS2) and *A. lipoferum* SpBr17 studied earlier have repeating-units with the same sugar composition but different structures (Chart 1, structures **1–3**, respectively).[15,16] These azospirilla were isolated from various soils in different climatic zones but all of them associate with cereals (*A. lipoferum* SR65 and *A. brasilense* S17 were isolated from the rhizosphere of wheat and rice, respectively, and *A. lipoferum* SpBr17 was isolated from surface-sterilized roots of maize). The similarity of their LPS may stem from the habitation of these bacteria in similar ecological niches, namely the cereal rhizospheres, where the living conditions are determined by the exudates from the plants. The azospirilla O-polysaccharides are directly implicated in endosymbiosis or root-surface colonization, and it is not excluded that their fine structures play a role in a particular bacterial–plant–root interaction mechanism.

1. Experimental

1.1. Bacterial strain, growth, isolation, and degradation of the lipopolysaccharide

A. lipoferum SR65, isolated from the roots of wheat (*Triticum aestivum* L., cv. Saratovskaya 40), was obtained from the microbial-culture collection held at the Institute of Biochemistry and Physiology of Plants and Microorganisms, RAS (Saratov). The culture was continuously grown in a 10-L ANKUM-2M fermentor at 30 °C in a liquid malate medium[7] to late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (10 g) were extracted with phenol–water,[17] and the isolated LPS was purified by repeated ultracentrifugation (105,000*g*, 2 × 4 h). The LPS yield was 2.3% of the dry cell weight.

An LPS sample (100 mg) was hydrolyzed with aq 2% HOAc at 100 °C for 5 h, the lipid precipitate (10.1 mg) was removed by centrifugation (13,000*g*, 20 min), and the carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer (pH 4.5) monitored with a Knauer differential refractometer. The yield of the PS was 34.5% of the LPS weight.

1.2. Chemical analyses and methylation

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h). The monosaccharides were analyzed by GLC as the alditol acetates[18] on an HP-5 capillary column by using a Hewlett–Packard 5890 instrument and a temperature gradient of 160 °C (1 min) to 290 °C at 7 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol, as described.[19]

The PS was methylated with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[20] The methylated PS was hydrolyzed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with an HP-5ms column, by using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.3. Smith degradation

A PS sample (20 mg) was oxidized with 0.1 M $NaIO_4$ (1.5 ml) in the dark at 20 °C for 48 h. After addition of an excess of ethylene

glycol, reduction with $NaBH_4$, and desalting on a column (80 × 1.6 cm) of TSK HW-40 (S) in water, the product was hydrolyzed with aq 2% AcOH at 100 °C for 2 h, reduced with $NaBH_4$, and desalted with a KU-2 cation-exchange resin (H^+-form). A modified PS (10 mg) was isolated by GPC on TSK HW-40 (S) in water.

1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from D_2O. ^1H and ^{13}C NMR spectra were recorded using Bruker DRX-500 instrument at 313 K for OPS and 303 K for DPS. 3-Trimethylsilylpropanoate-d_4 (δ_H 0.0 ppm) and acetone (δ_C 31.45 ppm) were taken as the internal standards. 2D experiments were performed using standard Bruker software. The TOCSY spectra were recorded with 200 ms duration of MLEV-17 spin-lock, the ROESY spectra were recorded with 200 ms duration of spin-lock. The HMBC spectrum was recorded with 60 ms delay for evolution of long-range couplings.

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References

1. Somers, E.; Vanderleyden, J.; Srinivasan, M. *Crit. Rev. Microbiol.* **2004**, *30*, 205–240.
2. Bashan, Y.; Holguin, G.; de-Bashan, L. E. *Can. J. Microbiol.* **2004**, *50*, 521–577.
3. Pinheiro, R. O.; Baldani, J. I.; Boddey, R. M. *Abstr. 9th Intern. Congress on Nitrogen Fixation*, Cancum, Mexico, 1992; p 157.
4. Yegorenkova, I. V.; Konnova, S. A.; Sachuk, V. N.; Ignatov, V. V. *Plant Soil* **2001**, *231*, 275–282.
5. Konnova, S. A.; Fedonenko, Yu. P.; Makarov, O. E.; Ignatov, V. V. *Biol. Bull.* **2003**, *30*, 354–360.
6. Fedonenko, Yu. P.; Egorenkova, I. V.; Konnova, S. A.; Ignatov, V. V. *Microbiologya* **2001**, *70*, 329–334.
7. Jofre, E.; Lagares, A.; Mori, G. *FEMS Microbiol. Lett.* **2004**, *231*, 267–275.
8. Fischer, S. E.; Miguel, M. J.; Mori, G. B. *FEMS Microbiol. Lett.* **2003**, *219*, 53–62.
9. Fedonenko, Yu. P.; Zatonsky, G. V.; Konnova, S. A.; Zdorovenko, E. L.; Ignatov, V. V. *Carbohydr. Res.* **2002**, *337*, 869–872.
10. Fedonenko, Yu. P.; Borisov, I. V.; Konnova, O. N.; Zdorovenko, E. L.; Katsy, E. I.; Konnova, S. A.; Ignatov, V. V. *Microbiologya* **2005**, *74*, 626–632.
11. Konnova, O. N.; Burygin, G. L.; Fedonenko, Yu. P.; Matora, L. Yu.; Pankin, K. E.; Konnova, S. A.; Ignatov, V. V. *Microbiologya* **2006**, *75*, 323–328.
12. Fedonenko, Yu. P.; Konnova, O. N.; Zatonsky, G. V.; Shashkov, A. S.; Konnova, S. A.; Zdorovenko, E. L.; Ignatov, V. V.; Knirel, Yu. A. *Carbohydr. Res.* **2004**, *339*, 1813–1816.
13. Fedonenko, Yu. P.; Konnova, O. N.; Zatonsky, G. V.; Konnova, S. A.; Kocharova, N. A.; Zdorovenko, E. L.; Ignatov, V. V. *Carbohydr. Res.* **2005**, *340*, 1259–1263.
14. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
15. Choma, A.; Lorkiewicz, Z.; Russa, R. *Abstr. 9th Intern. Congress on Nitrogen Fixation*, Cancum, Mexico, 1992; p 125.
16. Fedonenko, Yu. P.; Konnova, O. N.; Zdorovenko, E. L.; Konnova, S. A.; Zatonsky, G. V.; Shashkov, A. S.; Ignatov, V. V.; Knirel, Yu. A. *Carbohydr. Res.* **2008**, *343*, 810–816.
17. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
18. Sawardeker, J. S.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1603.
19. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
20. Conrad, H. E. *Methods Carbohydr. Chem.* **1972**, *6*, 361–364.