Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 1259–1263
Carbohydrate RESEARCH

Note

Structure of the O-polysaccharide from the *Azospirillum lipoferum* Sp59b lipopolysaccharide

Yuliya P. Fedonenko,[a] Olga N. Konnova,[a] George V. Zatonsky,[b] Svetlana A. Konnova,[a] Nina A. Kocharova,[b] Evelina L. Zdorovenko[b,*] and 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*

Received 17 November 2004; accepted 20 January 2005

Abstract—A neutral O-specific polysaccharide was obtained by mild acid hydrolysis of the lipopolysaccharide of the plant-growth-promoting bacterium *Azospirillum lipoferum* Sp59b. On the basis of sugar and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, including a NOESY experiment, the following structure of the branched hexasaccharide repeating unit of the O-polysaccharide was established:

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→

Keywords: Lipopolysaccharide; O-polysaccharide structure; Asymbiotic diazotroph; *Azospirillum lipoferum*

Bacteria of the genus *Azospirillum* are nitrogen-fixing organisms that live in association with different plants in the rhizosphere. Inoculation of *Azospirillum* results in a positive effect on plant development under appropriate growth conditions.[1] Up to now, seven species have been differentiated within the genus *Azospirillum*. Bacterial surface macromolecules such as exopolysaccharide, capsular polysaccharide and lipopolysaccharide (LPS) are involved in the associative *Azospirillum*–plant interaction.[2–6] Studies of these macromolecules, including chemical structure elucidation, are important for understanding at a molecular level the mechanisms that underlie the formation of effective plant–bacteria associations. Serological studies of LPS of azospirilla have been reported,[7,8] but, in contrast to many other Gram-negative bacteria, no LPS-based serological classification has been developed.

The structures of the O-polysaccharides of LPS, which are the major bacterial-surface antigens, have been determined only for three *Azospirillum* strains: *A. lipoferum* SpBr17,[9] *A. brasilense* Sp245,[10] *A. irakense* KBC1.[11] Therefore, studies on the structures of *Azospirillum* O-antigens remain topical. *A. lipoferum* is the first *Azospirillum* species described and also one of the most studied. In this paper, we report on the O-polysaccharide structure of the LPS from *A. lipoferum* Sp59b, a type strain of this species.

An (*S*)-type LPS sample was obtained in a yield of 2.6% from the water phase of the hot phenol–water extract of *A. lipoferum* Sp59b cells. Mild acid hydrolysis

* Corresponding author. Tel.: +7 095 9383613; fax: +7 095 1355328; e-mail: evelina@ioc.ac.ru

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.01.042

of the LPS afforded a lipid sediment and a water-soluble carbohydrate portion, which was fractionated by GPC on Sephadex G-50 to give a high-molecular-mass O-polysaccharide. Monosaccharide analysis by GLC of the alditol acetates obtained after complete acid hydrolysis of the polysaccharide revealed rhamnose, galactose and mannose in the ratios ~3:2:1, respectively. Determination of the absolute configurations by GLC of the acetylated glycosides with a chiral alcohol indicated that galactose and mannose have the D configuration, whereas rhamnose has the L configuration.

Methylation analysis of the polysaccharide, including GLC of the partially methylated alditol acetates, revealed derivatives from terminal, 2-substituted and 3-substituted rhamnose residues, 3-substituted and 3,4-disubstituted galactose residues and a 3-substituted mannose residue. These data showed that the O-polysaccharide is branched with a terminal rhamnose residue and a 3,4-disubstituted galactose residue at the branching point. They proved also the pyranose form of all monosaccharides, apart of the 3,4-disubstituted galactose residue, whose pyranose form was confirmed by NMR spectroscopic data (see below).

Each of the ^{1}H and ^{13}C NMR (Figs. 1 and 2) spectra of the polysaccharide contained signals for six monosaccharide residues and were assigned using 2D $^{1}H,^{1}H$ COSY, TOCSY, NOESY and H-detected $^{1}H,^{13}C$ HSQC experiments (Table 1). The spin systems for three Rha*p*, two Gal*p* and one Man*p* residues were elucidated from a combined analysis of COSY and TOCSY spectra. The assignment for one of the galactose residues (Galp^{I}) was completed by an H-1, H-5 correlation observed in the NOESY spectrum, which, together with a relatively large $J_{1,2}$ coupling constant of ~8 Hz, indicated the β-linkage of Gal^{I}. The α-linkage of the other galactose residue (Galp^{II}), which gave a nonsplit H-1 signal, was established by the C-1 chemical shift at δ 97.3 (a value for β-Gal*p*, α-Gal*f* or β-Gal*f* would be >δ 100).[12] The α-linkage of the three rhamnose residues (Rhap^{I}-Rhap^{III}) as well as the β-linkage of the mannose residue were determined by comparison of the C-5 chemical shifts δ 70.3–70.7 and 77.3, respectively (Table 1), with those of the corresponding α- and β-Man*p* (δ 73.7 and 77.4), α- and β-Rha*p* (δ 69.5 and 73.2).[12]

The ^{13}C NMR chemical shift data (Table 1) revealed low-field positions at δ 78.2, 79.6, 80.6, 76.9, 79.3 and 80.0 of the signals for Man C-3, Gal^{I} C-3, Gal^{II} C-3 and C-4, Rha^{I} C-3, and Rha^{II} C-2, respectively, as compared with their positions in the corresponding nonsubstituted monosaccharides.[12] These displacements by 5–9 ppm were due to glycosylation effects and defined the linkage pattern of the polysaccharide.

In addition to intraglycosidic correlation (H-1/H-2 for α-linked sugars and H-1/H-3, H-1/H-5 for β-linked sugars), the NOESY spectrum showed the following transglycosidic cross-peaks: Rha^{III} H-1, Rha^{II} H-2 at δ 5.07/4.04; Rha^{II} H-1, Rha^{I} H-3 at δ 5.11/3.96, Rha^{I} H-1,Man H-3 at δ 4.98/3.72, Man H-1, Gal^{II} H-4 at δ 5.12/4.54,

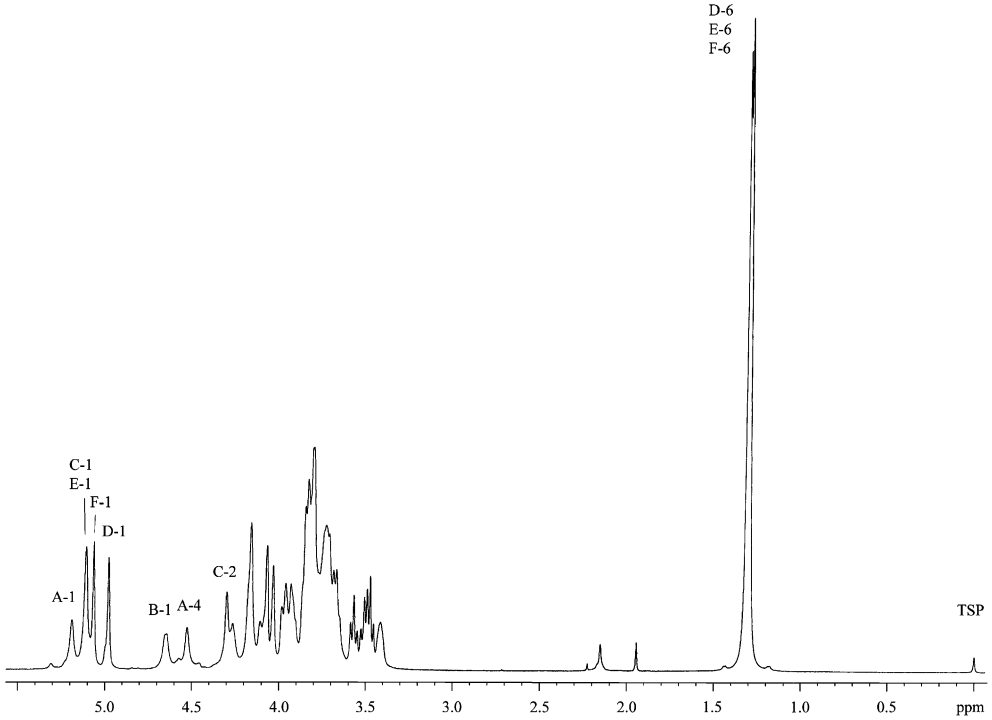

Figure 1. 500-MHz ^{1}H NMR spectrum of the O-polysaccharide of *A. lipoferum* Sp59b, where A is α-D-Galp^{II}, B—β-D-Galp^{I}, C—β-D-Man*p*, D—α-L-Rhap^{I}, E—α-L-Rhap^{II} and F—α-L-Rhap^{III}.

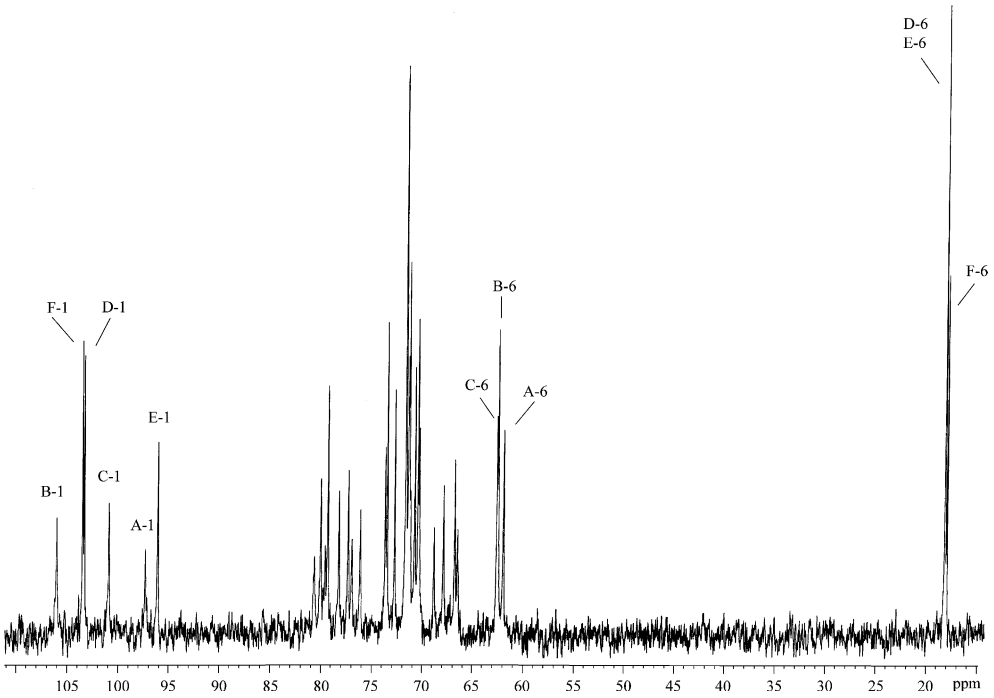

Figure 2. 125-MHz ^{13}C NMR spectrum of the O-polysaccharide of *A. lipoferum* Sp59b, where A is α-D-Gal*p*^{II}, B—β-D-Gal*p*^{I}, C—β-D-Man*p*, D—α-L-Rha*p*^{I}, E—α-L-Rha*p*^{II} and F—α-L-Rha*p*^{III}.

Table 1. 500-MHz ^{1}H NMR and 125-MHz ^{13}C NMR data of the O-polysaccharide (δ, ppm)

Monosaccharide residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→3,4)-α-D-Gal*p*^{II}-(1→	5.20	4.10	4.18	4.54	4.27	3.72	3.83
→3)-β-D-Gal*p*^{I}-(1→	4.65	3.74	3.74	4.12	3.68	3.80	3.80
→3)-β-D-Man*p*-(1→	5.12	4.31	3.72	3.67	3.42	3.77	3.94
→3)-α-L-Rha*p*^{I}-(1→	4.98	4.16	3.96	3.58	3.82	1.31	
→2)-α-L-Rha*p*^{II}-(1→	5.11	4.04	3.98	3.52	3.92	1.31	
α-L-Rha*p*^{III}-(1→	5.07	4.07	3.84	3.48	3.84	1.31	
	C-1	C-2	C-3	C-4	C-5	C-6	
→3,4)-α-D-Gal*p*^{II}-(1→	97.3	68.8	80.6	76.9	71.6	61.9	
→3)-β-D-Gal*p*^{I}-(1→	106.1	71.2	79.6	66.5	76.1	62.5	
→3)-β-D-Man*p*-(1→	100.9	67.9	78.2	66.8	77.3	62.6	
→3)-α-L-Rha*p*^{I}-(1→	103.3	71.3	79.3	72.7	70.7	18.2	
→2)-α-L-Rha*p*^{II}-(1→	96.1	80.0	71.4	73.6	70.3	18.2	
α-L-Rha*p*^{III}-(1→	103.5	71.6	71.6	73.4	70.4	18.0	

Gal^{II} H-1, Gal^{I} H-3 at δ 5.20/3.74 and Gal^{I} H-1, Gal^{II} H-3 at δ 4.65/4.18. These data confirmed the linkage pattern and defined the sequence of the sugar residues in the repeating unit.

On the basis of the data obtained, the structure in Figure 3 was established for the hexasaccharide repeating unit of the O-polysaccharide of *A. lipoferum* Sp59b. Recently, we have determined the structure of the O-polysaccharide of a strain of another *Azospirillum* species, *A. irakense* KBC1 (Fig. 3).[11] Remarkably, although structurally different, the repeating units of the two *Azospirillum* O-polysaccharides share the monosaccharide composition and a rather uncommon topology with a disaccharide repeat in the main chain and a tetrasaccharide side chain.

1. Experimental

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

Strain *A. lipoferum* Sp59b (VKM B-1519) isolated from wheat roots[13] was obtained from the All-Russian Collection of Microorganisms (VKM). The culture was continuously grown in a 10-L ANKUM-2M fermenter at

A. lipoferum Sp59b (this work)

α-L-Rha*p*^{III}-(1→3)-α-L-Rha*p*^{II}-(1→2)-α-L-Rha*p*^{I}-(1→3)-β-D-Man*p*-(1→4)┐
→3)-β-D-Gal*p*^{II}-(1→3)-α-D-Gal*p*^{I}-(1→

A. irakense KBC1[11]

α-D-Gal*f*-(1→2)-α-L-Rha*p*-(1→3)-β-D-Man*p*-(1→3)-α-L-Rha*p*-(1→3)┐
→3)-β-D-Gal*p*-(1→4)-α-L-Rha*p*-(1→

Figure 3. Structures of the O-polysaccharides of *Azospirillum lipoferum* Sp59b and *Azospirillum irakense* KBC1.

30 °C in a liquid malate medium[14] to the late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (10 g) were extracted with phenol–water,[15] and the LPS was purified by GPC on a column (55 × 1.8 cm) of Sepharose CL-4B in 0.025 M NH_4HCO_3 pH 8.3. The yield of the LPS was 2.6% of the dry cells weight.

1.2. Degradation of the lipopolysaccharide

A LPS sample (110 mg) was hydrolysed with aq 1% AcOH at 100 °C for 4 h, the lipid precipitate (14.5 mg) was removed by centrifugation (13,000*g*, 20 min), 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 with monitoring by a Knauer differential refractometer to give a high-molecular-mass polysaccharide (55 mg).

1.3. Monosaccharide analysis

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were analysed by GLC as the alditol acetates[16] on an Ultra 2 capillary column using a Hewlett-Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min^{-1}. The absolute configurations were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[17]

1.4. Methylation analysis

Methylation of the polysaccharide was carried out with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide.[18] Hydrolysis of the methylated polysaccharide was performed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Hewlett-Packard HP 5989A instrument equipped with an HP-5ms column using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.5. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 27 °C. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and external acetone (δ_C 30.45). A mixing time of 200 and 150 ms was used in TOCSY and NOESY experiments, respectively.

Acknowledgements

Authors thank Dr. A. S. Shashkov for help with interpretation of the NMR spectra and Dr. Y. A. Knirel for critical reading of the manuscript. This work was funded in part by the Foundation for Leading Scientific Schools of the Russian Federation (projects NSh-1557.2003.3 and NSh-1529.2003.4).

References

1. Okon, Y.; Vanderleyden, J. *ASM News* **1997**, *63*, 366–370.
2. De Troch, P.; Vanderleyden, J. *Microbiol. Ecol.* **1996**, *32*, 149–169.
3. Burdman, S.; Okon, Y.; Jurkevitch, E. *Crit. Rev. Microbiol.* **2000**, *26*, 91–110.
4. Yegorenkova, I. V.; Konnova, S. A.; Sachuk, V. N.; Ignatov, V. V. *Plant Soil* **2001**, *231*, 275–282.
5. Fedonenko, Y. P.; Yegorenkova, I. V.; Konnova, S. A.; Ignatov, V. V. *Mikrobiologiya* **2001**, *70*, 384–390.
6. Jofre, E.; Lagares, A.; Mori, G. *FEMS Microbiol. Lett.* **2004**, *231*, 267–275.
7. Bogatyrev, V. A.; Dykman, L. A.; Matora, L. Yu.; Schwartsburd, B. I. *FEMS Microbiol. Lett.* **1992**, *75*, 115–118.
8. Schloter, M.; Hartmann, A. *Hybridoma* **1996**, *15*, 225–232.
9. Choma, A.; Lorkiewicz, Z.; Russa, R. *Abstr. 9th Intern. Congress on Nitrogen Fixation*; Cancum, Mexico, 1992; p 125.

10. Fedonenko, Yu. P.; Zatonsky, G. V.; Konnova, S. A.; Zdorovenko, E. L.; Ignatov, V. V. *Carbohydr. Res.* **2002**, *337*, 869–872.
11. 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.
12. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
13. Tarrand, J. J.; Krieg, N. R.; Döbereiner, J. *Can. J. Microbiol.* **1978**, *24*, 967–980.
14. Konnova, S. A.; Makarov, O. E.; Skvortsov, I. M.; Ignatov, V. V. *FEMS Microbiol. Lett.* **1994**, *118*, 93–94.
15. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
16. Sawardeker, J. S.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1603.
17. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
18. Conrad, H. E. *Methods Carbohydr. Chem.* **1972**, *6*, 361–364.