Carbohydrate Research 344 (2009) 1259–1262

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Isolation and structure elucidation of two different polysaccharides from the lipopolysaccharide of *Rahnella aquatilis* 33071^{T}

Evelina L. Zdorovenko [a,*], Ludmila D. Varbanets [b], George V. Zatonsky [a], Galina M. Zdorovenko [b], Alexander S. Shashkov [a], Yuriy A. Knirel [a]

[a] N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospeckt 47, 119991 Moscow, Russia
[b] D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Zabolotnogo 154, 03143 Kiev, Ukraine

ARTICLE INFO

Article history:
Received 4 March 2009
Received in revised form 10 April 2009
Accepted 14 April 2009
Available online 17 April 2009

Keywords:
Lipopolysaccharide
O-Specific polysaccharide
Bacterial polysaccharide structure
Rahnella aquatilis

ABSTRACT

Two different polysaccharides were obtained by mild acid degradation of the lipopolysaccharide of *Rahnella aquatilis* 33071^{T}. These were studied by sugar and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The following structures were established for the polysaccharides:

→4)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-β-D-Man*p*-(1→

→3)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→

β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→4)-α-D-Glc*p*A-(1→2)-┘

The former structure is new, whereas the latter has been reported earlier as the structure of the O-specific polysaccharide of *R. aquatilis* 95 U003 (Zdorovenko, E. L.; Varbanets, L. D.; Zatonsky, G. V.; Kachala, V. V.; Zdorovenko, G. M.; Shashkov, A. S.; Knirel, Y. A. *Carbohydr. Res.* **2008**, *343*, 2494–2497).

Microorganisms of the family Enterobacteriaceae are an integral part of the biosphere. Owing to their polybiothrophy and adaptation plasticity, they are widespread in objects of the abiotic environment and are found in organisms in the flora and fauna. These bacteria essentially influence various spheres of human activity. Recently, a number of new genera and species were discovered in the family due to the improvement of traditional methodologies and development of new methods in taxonomy. However, a diversity of biological properties of the bacteria significantly complicates the identification of representatives of Enterobacteriaceae, especially strains of new (firstly described) and poorly studied species.

Rahnella aquatilis[1,2] is among more than 80 species that were recognized within the family Enterobacteriaceae but remained practically unexplored before 1995. These microorganisms are isolated from environmental objects, such as water reservoirs and soil (mostly the rhizospheres of the cereals,[3,4] corn, wheat and rice), and from clinical material.[5,6] *R. aquatilis* is a heterogeneous species, whose systematic taxonomy has many unsettled questions. Composition and structure of the lipopolysaccharide is one of the recognized chemotaxonomic criteria of Gram-negative bacteria, and its O-specific polysaccharide chain, called O-antigen, serves as the basis for the serological classification of bacterial strains. Earlier, we have elucidated structures of the O-specific polysaccharides of a number of *R. aquatilis* strains.[7–10] In this work, we obtained two different polysaccharides from the lipopolysaccharide of the type strain of the species *R. aquatilis* 33071^{T} and established their structures.

The lipopolysaccharide was isolated from dried bacterial cells of *R. aquatilis* 33071^{T} by phenol–water extraction. Mild acid degradation of the lipopolysaccharide, followed by GPC on Sephadex G-50, resulted in two polysaccharides (PSI and PSII).

Sugar analysis by GLC of the alditol acetates obtained after acid hydrolysis of PSI revealed glucose, mannose and galactose in the ratios 0.6:1:1. In addition, glucuronic acid (GlcA) was identified by anion-exchange chromatography of the hydrolysate using a sugar analyzer. Determination of the absolute configuration by GLC of the acetylated glycosides with (*S*)-2-octanol indicated that all monosaccharides have the D configuration. Methylation analysis of PSI, including GLC–MS of the partially methylated alditol acetates, revealed derivatives of 2,3,4,6-tetra-*O*-methylhexopyranose, 3,4,6-tri-*O*-methylhexopyranose, 2,4,6-tri-*O*-methylhexopyranose and 4,6-di-*O*-methylhexopyranose. In addition to these monosaccharides, after carboxyl reduction of the methylated polysaccharide, 2,3-di-*O*-methylhexose was identified, which was evidently derived from a 4-substituted GlcA residue.

* Corresponding author. Tel.: +7 499 1376148; fax: +7 499 1355328.
E-mail address: evelina@ioc.ac.ru (E.L. Zdorovenko).

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.04.013

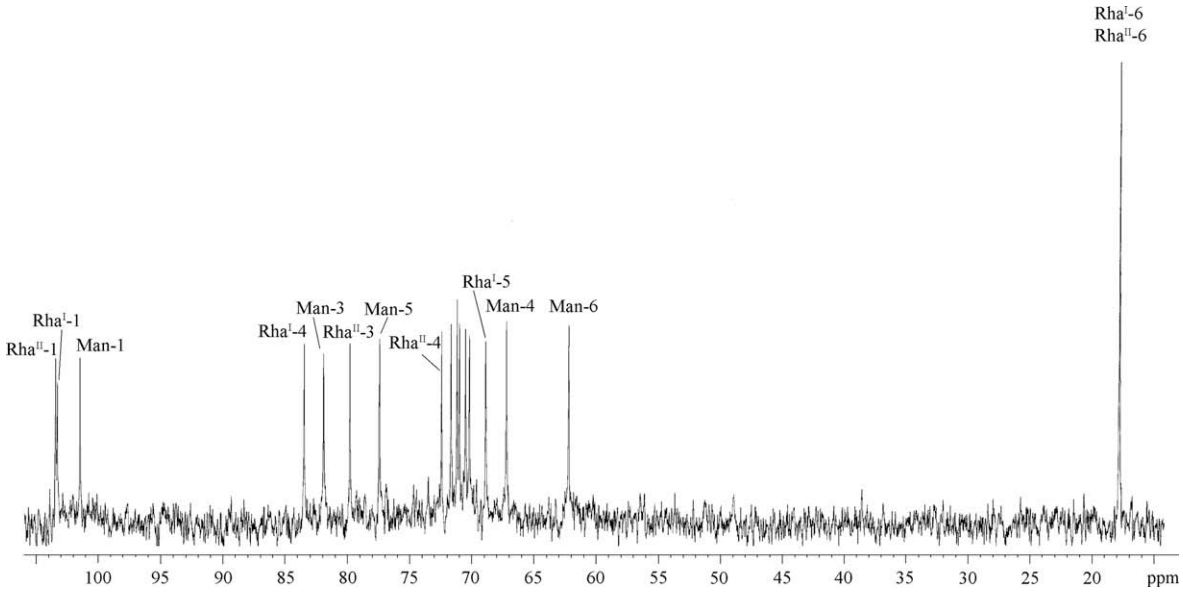

Figure 1. ^{13}C NMR spectrum of PSII from *R. aquatilis* 33071^{T}.

The ^{13}C NMR spectrum of PSI demonstrated a regular structure. It contained signals for six anomeric carbons at δ 96.4–105.1, five HO*C*H$_2$–C groups (C-6 of Man, Glc and Gal) at δ 62.2–62.7, one carboxyl group (C-6 of GlcA) at δ 174.6 and sugar ring carbons in the region δ 66.2–80.6. Accordingly, the ^{1}H NMR spectrum of PSI contained signals for six anomeric protons at δ 4.61–5.46 and other sugar protons in the region of δ 3.30–4.23. The NMR spectra of PSI were remarkably similar to those of the O-specific polysaccharide of *R. aquatilis* 95 U003, whose structure has been established earlier.[9] Based on these similarities, combined with the data from both the compositional and methylation analyses, PSI from *R. aquatilis* 33071^{T} has the same structure as the polysaccharide of *R. aquatilis* 95 U003:

$$\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow$$
$$\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 2)\rfloor$$

Sugar analysis and determination of the absolute configuration of the monosaccharides from PSII revealed D-rhamnose and D-mannose in the ratio 1:1 (detector response). Methylation analysis showed the presence of 2,4-di-*O*-methylrhamnose, 2,3-di-*O*-methylrhamnose and 2,4,6-tri-*O*-methyl-mannose, as well as minor components, which were evidently derived from the lipopolysaccharide core and a PSI contamination. Therefore, PSII is linear.

The ^{13}C NMR spectrum of PSII contained signals for three anomeric carbons at δ 101.5–103.4, two *C*H$_3$–C groups (C-6 of Rha) at δ 17.9, one HO*C*H$_2$–C group (C-6 of Man) at δ 62.3 and 12 sugar ring carbons in the region δ 67.2–83.5. The absence of signals from the region of δ 84–88 characteristic of furanosides[11] showed that all monosaccharide residues are in the pyranose form. The ^{1}H NMR spectrum of PSII contained inter alia three signals at δ 4.80–5.07 for anomeric protons and two signals for CH$_3$–C groups (H-6 of Rha) at δ 1.30 and 1.32. These data confirmed the composition of PSII as determined by sugar and methylation analyses. A higher than expected content of mannose detected in sugar analysis could be accounted for by partial destruction of rhamnose during acid hydrolysis.

The chemical shifts in the ^{1}H and ^{13}C NMR spectra of PSII (Fig. 1) were assigned using 2D COSY, TOCSY, NOESY and ^{1}H,^{13}C HSQC experiments (Table 1), and spin systems for one mannose and two rhamnose residues (Rha^{I} and Rha^{II}) were revealed. The assignment was based on correlations of H-1 with H-2–H-4 and H-6 with H-5–H-2 for Rha^{I} and Rha^{II}, as well as H-1 with H-2 and H-2 with H-3–H-6 for Man in the TOCSY spectrum. The assignment within each spin system was performed using the COSY spectrum.

The β configuration of Man was established by the relatively low-field position of the C-5 signal at δ 77.5 in the ^{13}C NMR spectrum, and the α configuration of Rha^{I} and Rha^{II} by the relatively high-field positions of the C-5 signals at δ 68.9–70.5 (compare published data δ 69.4 and 73.1 for α- and β-rhamnopyranoses, respectively[12]). Relatively low-field positions of the signals for C-4 of Rha^{I}, C-3 of Rha^{II} and C-3 of Man at δ 83.5, 79.8 and 81.9, respectively, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides,[12] were in agreement with the substitution pattern determined by methylation analysis (see above).

The NOESY spectrum of PSII (Fig. 2) showed strong interresidue cross-peaks between the anomeric protons and protons at the linkage carbons, which were assigned taking into account the ^{13}C NMR chemical shift data (see above) as follows: H-1 Rha^{I}/H-3 Rha^{II}; H-1 Rha^{II}/H-3 Man; and H-1 Man/H-4 Rha^{I}, at δ 5.07/3.92, 5.04/3.73, 4.80/3.66, respectively. These correlations revealed the monosaccharide sequence in the repeating unit. The presence of H-1/H-2 intraresidue cross-peaks confirmed the α configuration of Rha^{I} and Rha^{II}, and H-1/H-2; H-1/H-3 and H-1/H-5 cross-peaks were in accord with the β configuration of Man.

Table 1
500-MHz ^{1}H NMR and 125-MHz ^{13}C NMR data of PSII from *R. aquatilis* 33071^{T} (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a,6b
→4)-α-D-Rha*p*^{I}-(1→	5.07	4.14	3.97	3.66	3.95	1.32
→3)-α-D-Rha*p*^{II}-(1→	5.04	4.15	3.92	3.57	3.90	1.30
→3)-β-D-Man*p*-(1→	4.80	4.13	3.73	3.73	3.47	3.76, 3.95
	C-1	C-2	C-3	C-4	C-5	C-6
→4)-α-D-Rha*p*^{I}-(1→	103.3	71.0	70.2	83.5	68.9	17.9
→3)-α-D-Rha*p*^{II}-(1→	103.4	71.2	79.8	72.5	70.5	17.9
→3)-β-D-Man*p*-(1→	101.5	71.7	81.9	67.2	77.5	62.3

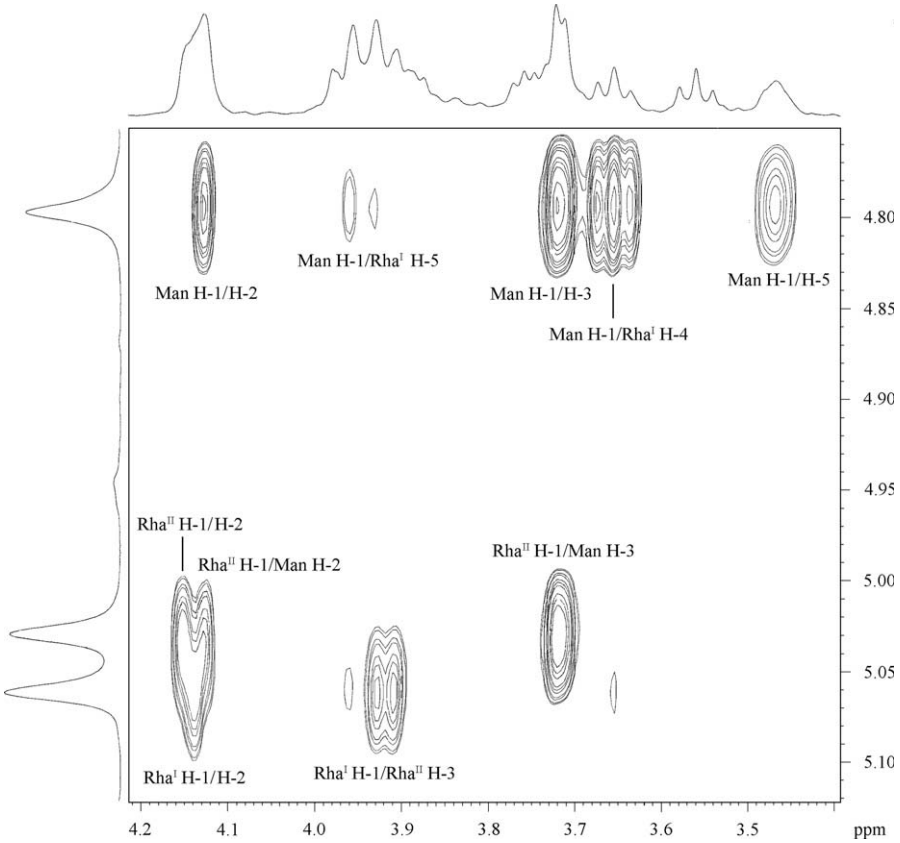

Figure 2. Part of a 2D NOESY spectrum of PSII from *R. aquatilis* 33071^{T}. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes.

Based on the data obtained, it was concluded that PSII from *R. aquatilis* 33071^{T} has the following structure, which to the best of our knowledge, has not been hitherto reported for any bacterial polysaccharide:

→ 4)-α-D-Rha*p*-(1 → 3)-α-D-Rha*p*-(1 → 3)-β-D-Man*p*-(1 →

1. Experimental

1.1. Growth of bacteria and isolation of the lipopolysaccharide and polysaccharides

R. aquatilis 33071^{T} isolated from excrements of a clinically healthy patient[5] was kindly provided by Dr. S. I. Pohyl (Institute of Microbiology and Immunology, Medical Academy of Sciences of Ukraine, Kharkov, Ukraine). The culture was grown at 28 °C for 24 h on a beef-extract agar medium, cells were separated by centrifugation, washed subsequently with acetone and ether and dried on air. The lipopolysaccharide isolated by the phenol–water procedure[13] was recovered from the aqueous layer and was purified by a three-step ultracentrifugation (105,000*g*, 4 h).

The polysaccharides were obtained by degradation of the lipopolysaccharide with 3% aq HOAc (100 °C, 3 h), followed by GPC on a column (70 × 3.0 cm) of Sephadex G-50 (S) using 0.05 M pyridine acetate pH 4.5 as eluent and monitoring by the phenol–sulfuric acid reaction. The yields of PSI and PSII were 30% and 25%, respectively.

1.2. Sugar analysis

Hydrolysis of the polysaccharides was performed with 2 M CF_3CO_2H (120 °C, 2 h). The monosaccharides were analyzed by GLC as the alditol acetates on an Ultra 2 capillary column (25 m × 0.2 mm, film thickness 0.33 μm) using a Hewlett–Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min^{-1}. A portion of the hydrolysate was analyzed using a Biotronik LC 2000 sugar analyzer equipped with a column (80 × 4 mm) of a DA × 8 anion-exchange resin (70 °C, 0.02 M KH_2PO_4–H_3PO_4 buffer, pH 2.9) and detection with the Cu-bicinchoninate reagent.[14] The absolute configurations were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[15]

1.3. Methylation analysis

Methylation of the polysaccharides was carried out with CH_3I in dimethyl sulfoxide in the presence of methylsulfinylmethanide.[16] A portion of the methylated PSI was reduced with $LiBH_4$ in 70% aq 2-PrOH (20 °C, 2 h). Hydrolysis of the methylated polysaccharide was performed with 2 M CF_3CO_2H (120 °C, 2 h), and the partially methylated monosaccharides were conventionally reduced with $NaBH_4$ and acetylated. GLC–MS analysis was performed 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}.

1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from 99.9% $^{2}H_2O$. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in 99.96% $^{2}H_2O$ at 50 °C. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and external acetone (δ_C 31.45). A mixing time of 150 and 200 ms was used in TOCSY and NOESY experiments, respectively.

Acknowledgements

The authors thank Dr. S. I. Pohyl for the bacterial culture. This work was supported by the Russian Foundation for Basic Research (Project 09-04-90446-Ukr_f_a) and by the Ukrainian State Foundation for Basic Research (Project F28.4/039).

References

1. Gavini, F.; Ferragut, C.; Lefebre, B.; Leclerc, H. *Ann. Microbiol. Inst. Pasteur* **1976**, *127*, 317–335.
2. Izard, D.; Gavini, F.; Frinel, P.; Leclerc, H. *Ann. Microbiol. Inst. Pasteur* **1979**, *130*, 163–167.
3. Kil, Y. K.; Diann, J.; Krishman, H. B. *FEMS Microbiol. Lett.* **1997**, *153*, 273–277.
4. Berge, O.; Heulin, T.; Achouak, W. *Can. J. Microbiol.* **1991**, *37*, 195–203.
5. Pokhil, S. I. *Mikrobiol. Zh.* **1998**, *60*, 31–37.
6. Reina, J.; Lopez, A. *J. Infect.* **1996**, *33*, 135–137.
7. Zdorovenko, E. L.; Varbanets, L. D.; Zatonsky, G. V.; Ostapchuk, A. N. *Carbohydr. Res.* **2004**, *339*, 1809–1812.
8. Zdorovenko, E. L.; Varbanets, L. D.; Zatonsky, G. V.; Ostapchuk, A. N. *Carbohydr. Res.* **2006**, *341*, 164–168.
9. Zdorovenko, E. L.; Varbanets, L. D.; Zatonsky, G. V.; Kachala, V. V.; Zdorovenko, G. M.; Shashkov, A. S.; Knirel, Y. A. *Carbohydr. Res.* **2008**, *343*, 2494–2497.
10. Varbanets, L. D.; Zdorovenko, E. L.; Ostapchuk, A. N. *Mikrobiologiia* **2008**, *77*, 298–304.
11. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
12. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59–75.
13. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
14. Sinner, M.; Puls, J. *J. Chromatogr.* **1978**, *156*, 197–204.
15. Leontein, K.; Lönngren, J. *Methods Carbohydr. Chem.* **1993**, *9*, 87–89.
16. Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.