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Carbohydrate Research 341 (2006) 164–168
Carbohydrate RESEARCH

Note

Structures of two putative O-specific polysaccharides from the *Rahnella aquatilis* 3-95 lipopolysaccharide

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Received 3 October 2005; accepted 9 November 2005
Available online 28 November 2005

Abstract—Two polysaccharide preparations (OPSI and OPSII) were obtained by mild acid degradation of the lipopolysaccharide of *Rahnella aquatilis* 3-95. Studies by chemical methods and ^{1}H and ^{13}C NMR spectroscopy showed that OPSI is a linear α-D-mannan having a trisaccharide repeat and OPSII is a ~2:1 mixture of the same mannan and an α-D-glucan:

→2)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→6)-α-D-Man*p*-(1→

→6)-α-D-Glc*p*-(1→

Keywords: Lipopolysaccharide; O-Antigen; Bacterial polysaccharide structure; *Rahnella aquatilis*

Recently as a result of onrush improvement of the traditional methodologies and development of new methods in taxonomy, a number of new genera and species were discovered in the family Enterobacteriaceae. However, from over 100 species of enterobacteria identified before 1995 only few draw attention of investigators, whereas the others, including *Rahnella aquatilis*, remained practically unexplored. Formerly, *R. aquatilis* was assigned to *Enterobacter agglomerans* biogroup G1. In 1976, using the principle of numeric taxonomy and having evaluated the phenotypic characteristics, a specific group of bacteria designated as H2 was recognized in the genus *Enterobacter*.[1] DNA–DNA hybridization showed that these bacteria should be singled out as a distinct species named *R. aquatilis*.[2] One of the recognized criteria used in the taxonomy of Gram-negative bacteria is the composition and structure of the lipopolysaccharide (LPS). No chemical data on the *R. aquatilis* LPS had been available until the structure of the O-polysaccharide of *R. aquatilis* 1-95 was reported in 2004.[3] Now, we report on two polysaccharides isolated from the LPS of *R. aquatilis* 3-95.

LPS of *R. aquatilis* 3-95 was isolated by phenol–water extraction.[4] LPS from the water fraction was used for analysis. The yield of LPS was 5.3% from the dry cell weight. The LPS preparation was purified by three-step ultracentrifugation. A sediment recovered by ultracentrifugation was used for further investigation. Compositional analyses of the LPS showed the presence of Man, Gal and Glc in the ratio 9:2:1. Gal was not found in the O-polysaccharide and, most likely, is a component of the core oligosaccharide.

Mild acid degradation of the LPS of *R. aquatilis* 3-95 resulted in two high-molecular-mass polysaccharide

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doi:10.1016/j.carres.2005.11.005

preparations (OPSI and OPSII), which were isolated by GPC on Sephadex G-50. Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the polysaccharides revealed mannose in OPSI and mannose and glucose in a ~6:1 ratio in OPSII. Determination of the absolute configuration by GLC of the acetylated (*S*)-2-octyl glycosides indicated that both monosaccharides have the D configuration. Methylation analysis of OPSI, including GLC of the partially methylated alditol acetates, revealed derivatives of 2,3,4-tri-*O*-methylmannopyranose, 3,4,6-tri-*O*-methylmannopyranose and 2,4,6-tri-*O*-methylmannopyranose in the ratio ~1.2:1:0.3, and mass spectrum of OPSII contained additional peak of 2,3,4-tri-*O*-methylglucopyranose. Therefore, OPSI is a linear homopolymer of mannose.

The ^{13}C NMR spectrum of OPSI (Fig. 1) contained signals for three anomeric carbons at δ 101.0–103.5, three CH_2O-groups (Man C-6) at δ 62.2, 62.4 and 66.3 and 12 other sugar carbons in the region δ 67.5–79.3. Accordingly, the ^1H NMR spectrum of OPSI (Fig. 2) contained signals for three anomeric protons at 4.92–5.32 and other sugar protons in the region δ 3.65–4.14. Therefore, OPSI has a trisaccharide repeating unit. The ^1H and ^{13}C NMR spectra of OPSI were assigned using 2D ^1H, ^1H COSY, TOCSY, NOESY and H-detected ^1H, ^{13}C HSQC experiments (Table 1). Spin systems for three Man*p* residues (Man^I–Man^{III}) were isolated by tracing connectivities in the TOCSY spectrum starting from H-1. The assignment within each spin system was performed using the COSY data.

Low-field displacements to δ 79.3, 78.7 and 66.3 of the signals for C-2, C-3 and C-6 of Man^I, Man^{II} and Man^{III}, as compared with their positions in non-substituted α-mannopyranose at δ 71.7, 71.3, 62.1, respectively,[5] showed that OPSI is linear and revealed the positions of substitution of each monosaccharide residue. A NOESY experiment revealed the following interresidue correlations between the anomeric protons and protons at the linkage carbons: Man^{III} H-1,Man^I H-2 and H-3, Man^I H-1,Man^{II} H-3 and Man^{II} H-1,Man^{III} H-6a,b at δ 5.07/4.10 and 4.07, 5.32/3.86 and 4.92/3.91 and 3.79, respectively. These data defined the monosaccharide sequence in the repeating unit.

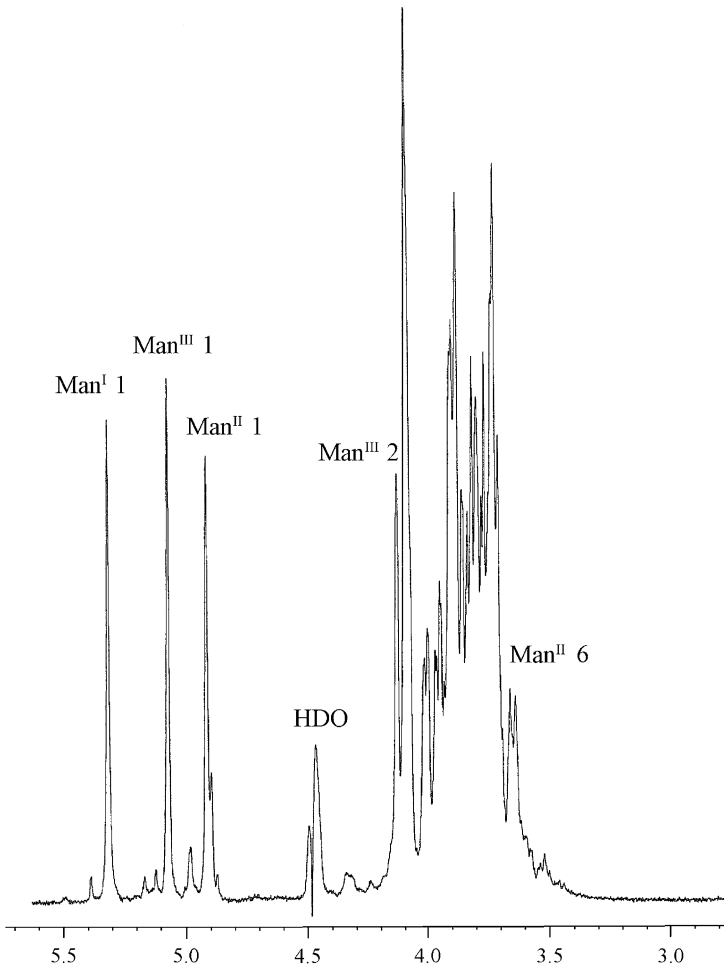

Figure 1. 500-MHz ^1H NMR spectrum of OPSI from the lipopolysaccharide of *R. aquatilis* 3-95.

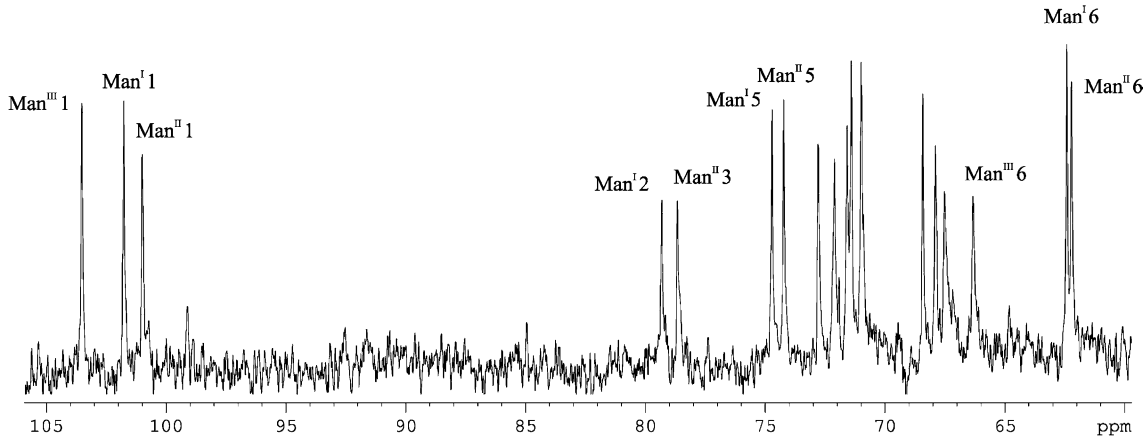

Figure 2. 125-MHz ^{13}C NMR spectrum of OPSI from the lipopolysaccharide of *R. aquatilis* 3-95.

Table 1. Data of 500-MHz ^{1}H NMR spectra and 125-MHz ^{13}C NMR spectra (δ, ppm)

Sugar residue	1	2	3	4	5	6(6a)	6b
OPSI							
→2)-α-D-Man*p*^{I}-(1→	5.32	4.10	4.07	3.73	3.76	3.90	3.77
	101.8	79.3	71.9	68.4	74.7	62.4	
→3)-α-D-Man*p*^{II}-(1→	4.92	4.04	3.86	3.93	3.82	4.04	3.65
	101.0	71.0	78.7	67.9	74.2	62.2	
→6)-α-D-Man*p*^{III}-(1→	5.07	4.14	3.97	3.83	3.73	3.91	3.79
	103.5	71.4	72.1	67.5	72.8	66.3	
OPSII							
→6)-α-D-Glc*p*-(1→	4.98	3.58	3.78	3.53	3.92	3.98	3.78
	99.2	73.0	74.2	71.1	71.7	67.2	

Therefore, OPSI from the LPS of *R. aquatilis* 3-95 has the following structure:

for Man^{I}–Man^{III}, a spin system for α-Glc was recognized (Table 1). Taking together the NMR chemical

$$\rightarrow 2)\text{-}\alpha\text{-D-Man}p^{I}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p^{II}\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p^{III}\text{-}(1\rightarrow$$

Earlier D-mannans or main D-mannan chains of the O-polysaccharides have been found in a number of Gram-negative bacteria, including *Escherichia coli* O8,[6] O9,[7] *Klebsiella pneumoniae* O3,[8] *Hafnia alvei* PCM 1223,[9] *Citrobacter braakii* O7a,3b,1c,[10] *Acetobacter methanolicus*[11] and *Pseudomonas diminuta*.[12] However, the structure of the OPSI of *R. aquatilis* 3-95 is unique among bacterial O-polysaccharide structures reported so far.[13]

The ^{1}H NMR spectrum of OPSII (Fig. 3) contained the same three signals for anomeric protons at δ 4.92, 5.07 and 5.32 as the spectrum of OPSI, and an additional anomeric signal at δ 4.98. Accordingly, the ^{13}C NMR spectrum of the OPSII contained three signals from OPSI at δ 101.0, 101.8 and 103.5, and an additional anomeric signals at δ 99.2.

The ^{1}H and ^{13}C NMR spectra of OPSII were assigned using two-dimensional COSY and ^{1}H,^{13}C HSQC experiments, respectively, and, in addition to the spin systems shifts, NOESY data, sugar analysis data and methylation analysis data (see above), it was suggested that OPSII is a mixture of OPSI and a glucan having the following structure:

$$\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow$$

Glucans are known as non-specific polysaccharides produced by different groups of organisms, including plants, weeds, fungi, Gram-positive and Gram-negative bacteria.[14–22] Some of them are structural ingredients of the bacterial cell wall.[18,19] The presence of a glucan in the LPS preparation from *R. aquatilis* 3-95 could not be accounted for by co-extraction of reserve glucan since upon three-steps ultracentrifugation of the LPS preparation after extraction, it would then be recovered from the supernatant rather than as a sediment, for example, as a low-molecular mass glucan from *Yersinia pseudo-*

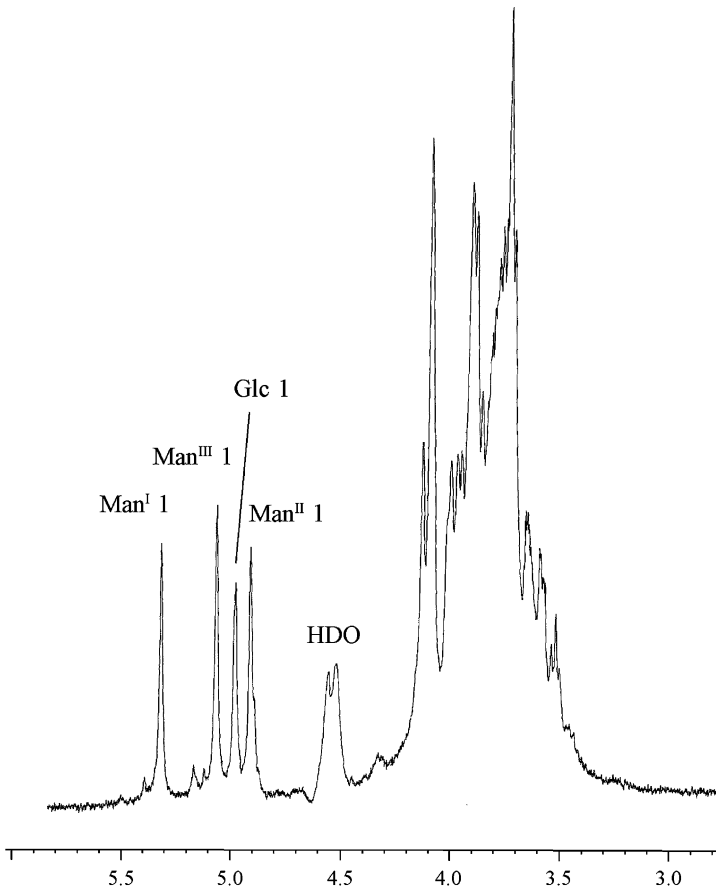

Figure 3. 500-MHz ^{1}H NMR spectrum of OPSII from the lipopolysaccharide of *R. aquatilis* 3-95.

tuberculosis serovar VI did.[23] Earlier, an LPS core-linked glucan having the same structure has been found in *Helicobacter pylori*.[24,25] Glucans of different structures have been found as a part of the LPS of *Pseudomonas fluorescens*[26] and *H. pylori* O2.[24]

Therefore, a mannan and a glucan were isolated from the LPS preparation from *R. aquatilis* 3-95, which both are suggested to be core-linked polysaccharide chains of the LPS.

1. Experimental

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

The culture of *R. aquatilis* 3-95 isolated from excrements of a patient child with an acute intestinal disease was kindly provided by Dr. S. Pohyl (Institute of Microbiology and Immunology, Medical Academy of Sciences of Ukraine, Kharkov, Ukraine) and was grown at 28 °C for 24 h on a beef-extract agar medium. Cells were separated by centrifugation and dried by acetone and ether in air. The LPS was isolated by the standard phenol–water procedure,[4] for analyses water fraction was used, followed by three-steps ultracentrifugation (105,000*g*, 4 h).

The polysaccharide preparations were obtained by degradation of the LPS with 3% aq AcOH (100 °C, 3 h) followed by GPC on a column (70 × 3.0 cm) of Sephadex G-50 (S) using 0.05 M pyridinium acetate pH 4.5 as eluent and monitoring by the phenol–sulfuric acid reaction.

1.2. Sugar analysis

Hydrolysis 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 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.[27]

1.3. Methylation analysis

Methylation of the polysaccharide was carried out with CH_3I in dimethyl sulfoxide in the presence of methylsulfinylmethanide.[28] Hydrolysis of the methylated

polysaccharide was performed with 2 M CF_3CO_2H (120 °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-5 ms column using a temperature gradient of 150 (3 min) to 320 °C at 5 °C min^{-1}.

1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from ^2H_2O. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in ^2H_2O at 27 °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 200 and 150 ms was used in TOCSY and NOESY experiments, respectively.

Acknowledgements

Authors thank Dr. S.I. Pohyl for the bacterial culture and Dr. Y.A. Knirel for critical reading of the manuscript. This work was supported in part by the Russian state contract RI-112/001/272.

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