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Structural and serological studies of the O-antigen of *Proteus mirabilis* O-9

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Abstract

The following structure of the O-polysaccharide (O-antigen) of the lipopolysaccharide of *Proteus mirabilis* O-9 was determined by NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, and $^{1}H,^{13}C$ HMQC experiments, along with chemical methods:

→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→
3
|
OAc

where the degree of O-acetylation is ~70%. Immunochemical studies using rabbit polyclonal anti-*Proteus mirabilis* O-9 serum showed the importance of the *O*-acetyl groups in manifesting the serological specificity of the O-9 antigen. Anti-*P. mirabilis* O-9 cross-reacted with the lipopolysaccharides (LPS) of *P. vulgaris* O-25 and *Proteus penneri* 14, which could be accounted for by a structural similarity of their O-polysaccharides.

Keywords: Lipopolysaccharide; O-Antigen; Bacterial polysaccharide; *Proteus mirabilis*; Structure; Ribose; O-Acetylation

1. Introduction

Proteus mirabilis is the most important *Proteus* human facultative pathogen. It frequently causes urinary tract and wound infections, which can lead to acute and chronic pyelonephritis, bacteriemia and formation of bladder and kidney stones.[1–4] It has also been suggested that *Proteus* surface antigens play a role in rheumatoid arthritis.[5–7] The lipopolysaccharide (LPS) of *Proteus* is considered as one of the virulent factors of the microorganism,[1] and the O-specific polysaccharide chain (O-polysaccharide, O-antigen) of the LPS defines the immunospecificity of the bacteria.

Based on the O-antigens, *Proteus* strains have been classified into more than 70 O-serogroups.[8–10] In most O-serogroups, the O-polysaccharide is acidic due to the presence of uronic acids, amino acids, phosphate groups or/and other acidic non-sugar components.[11] In this paper, we report on the structure of the O-polysaccharide of *P. mirabilis* O-9, which contains galacturonic acid and an O-acetylated ribofuranose residue, and on the involvement of the latter sugar in manifesting the serological specificity of the *P. mirabilis* O-9 antigen.

2. Results and discussion

The LPS was extracted from dried bacterial cells of *Proteus vulgaris* O-9 by the phenol–water procedure.[12] The yield of the purified LPS was 5.5% of dry bacterial mass. Mild acid degradation of the LPS with diluted acetic acid at 100 °C, followed by GPC on Sephadex G-50, resulted in a high-molecular-mass O-polysaccharide.

Monosaccharide analysis by GLC of the acetylated alditol acetates showed that the O-polysaccharide con-

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tains Rib, Gal, and GlcN. GLC of the acetylated (*S*)-2-octyl glycosides[13] showed the D configuration for all constituent monosaccharides. Methylation analysis revealed the presence of 2-substituted Rib*f*, 4-substituted Gal*p* (or 5-substituted Gal*f*) and 3-substituted GlcN. When the methylated polysaccharide was carboxyl-reduced with $LiBH_4$, a derivative of 2,3-di-*O*-methylgalactose was identified, which originated from 4-substituted GalA (see below).

The ^{13}C NMR spectrum of the O-polysaccharide (Fig. 1, Table 1) contained signals having different intensities, most likely, owing to non-stoichiometric O-acetylation (there were signals for CH_3 of an *O*-acetyl group at δ 21.3). The ^1H NMR spectrum of the O-polysaccharide (Fig. 2, Table 2) showed signals for *N*-acetyl and *O*-acetyl groups at δ 2.05 and 2.10 in the ratio approx 2:1, respectively.

The ^{13}C NMR spectrum of *O*-deacetylated O-polysaccharide (Table 1) contained signals for four anomeric carbons at δ 99.2, 100.3, 104.7, and 108.3, one nitrogen-bearing carbon at δ 53.9, three unsubstituted HO*C*H_2–C groups at δ 61.3, 62.4 and 63.8, and 14 oxygen-bearing carbons at δ 68.9–84.2. The signals at δ 23.6 (CH_3) and 175.7 (CO) indicated the presence of one *N*-acetyl group. The total number of signals in the spectrum and the presence of a signal at δ 84.2, which is typical of C-4 of a furanose,[14] confirmed the occurrence of a pentose (ribose) in the furanose form. The ^1H NMR spectrum of the O-deacetylated polysaccharide (Table 2) contained, inter alia, five signals at δ 4.48–5.41, which belonged to four anomeric protons and H-3 of ribofuranose (see below), and one signal for an *N*-acetyl group at δ 2.05. Therefore, the O-polysaccharide has a tetrasaccharide repeating unit containing one residue each of Gal, Rib, GalA and GlcNAc, and an *O*-acetyl group.

The ^1H and ^{13}C NMR spectra of the O-polysaccharide were assigned using 2D ^1H,^1H COSY, TOCSY, and H-detected ^1H,^{13}C HMQC experiments (Tables 1 and 2). The TOCSY spectrum showed that two signals in the low-field region of the ^1H NMR spectrum (δ 5.41 and 4.77) belonged to the same residue, which was identified as ribofuranose (Rib*f*). The spin system of Glc*p*NAc was identified on the basis of relatively large

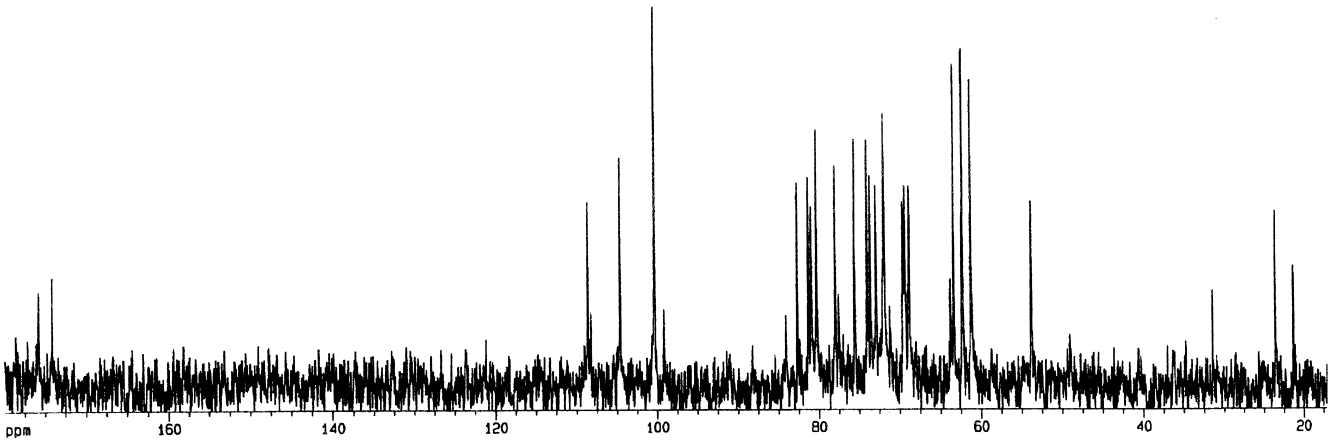

Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* O-9.

Table 1
^{13}C NMR data (δ, ppm) for the O-polysaccharide of *P. mirabilis* O-9

Residue	C-1	C-2	C-3	C-4	C-5	C-6
O-Deacetylated polysaccharide						
→4)-β-Gal*p*-(1→	104.7	71.3	74.0	77.6	75.6	62.4
→3)-α-Glc*p*NAc-(1→	100.3	53.9	80.9	69.4	71.9	61.3
→4)-α-Gal*p*A-(1→	99.2	68.9	69.4	80.0	71.6	178.6
→2)-β-Rib*f*-(1→	108.3	81.5	72.9	84.2	63.8	
Intact O-polysaccharide						
→4)-β-Gal*p*-(1→	104.7	71.9	73.9	77.9	75.6	62.3
→3)-α-Glc*p*NAc-(1→	100.3	53.8	80.9	69.6	72.9	61.3
→4)-α-Gal*p*A-(1→	100.3	68.8	69.6	80.3	71.9	174.1
→2)-β-Rib*f*-(1→	108.6	81.3	73.6	82.7	63.4	

Additional chemical shifts are: NAc δ 23.6 (Me), 175.7 (CO); OAc δ 21.3 (Me), 175.7 (CO).

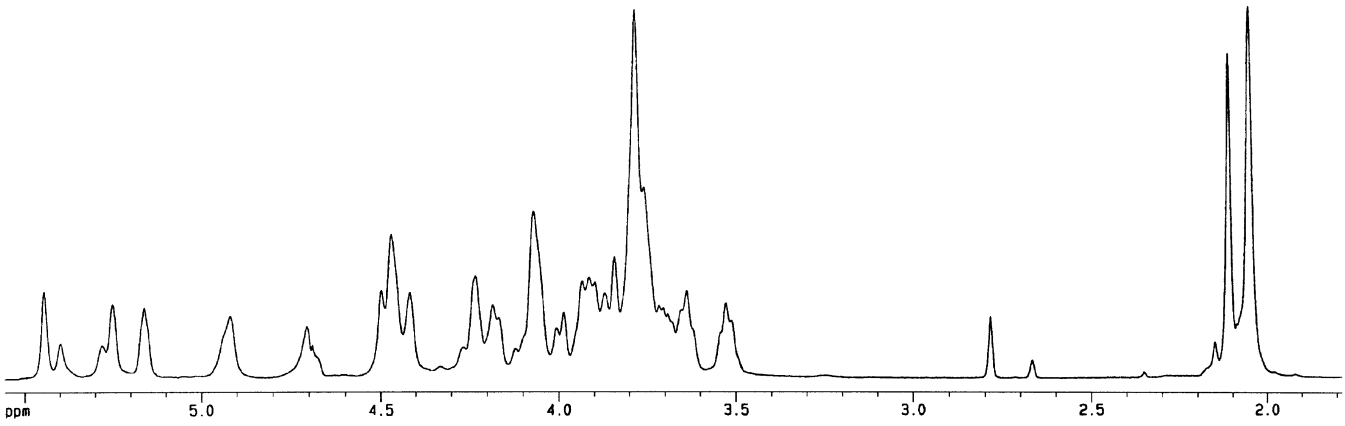

Fig. 2. ^{1}H NMR spectrum of the O-polysaccharide of *P. mirabilis* O-9.

Table 2
^{1}H NMR data (δ, ppm) for the O-polysaccharide of *P. mirabilis* O-9

Sugar residue	H-1	H-2	H-3	H-4	H-5 (H-5a,5b)	H-6 (H-6a,6b)
O-Deacetylated polysaccharide						
→4)-β-Gal*p*-(1→	4.48	3.52	3.80	4.06	3.76	3.78
→3)-α-Glc*p*NAc-(1→	4.95	4.07	3.90	3.67	4.22	3.87, 3.78
→4)-α-Gal*p*A-(1→	5.30	3.95	4.12	4.45	4.25	
→2)-β-Rib*f*-(1→	5.41	4.26	4.77	4.08	3.85, 3.69	
Intact O-polysaccharide						
→4)-β-Gal*p*-(1→	4.45	3.53	3.79	4.06	3.74	3.78
→3)-α-Glc*p*NAc-(1→	4.92	4.07	3.89	3.64	4.18	3.84, 3.78
→4)-α-Gal*p*A-(1→	5.25	3.94	4.00	4.42	4.50	
→2)-β-Rib*f*-(1→	5.43	4.47	5.15	4.24	3.80, 3.46	

Additional chemical shifts are: NAc δ 2.05; OAc δ 2.10.

$J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ coupling constant values ($\sim$10 Hz) and the correlation of the proton at a nitrogen-bearing carbon at δ 4.07 to the corresponding carbon at δ 53.9 observed in the ^{1}H,^{13}C HMQC spectrum. Two remaining spin systems, those of Gal*p* and Gal*p*A, where identified by relatively small $J_{3,4}$ and $J_{4,5}$ coupling constant values ($<$3 Hz) and distinguished from each other by the position and splitting pattern of the H-5 signal of β-Gal*p*, which was assigned by the H-1,H-5 correlation in the 2D ROESY spectrum. A $J_{1,2}$ coupling constant value of $\sim$3 Hz indicated that Gal*p*A and Glc*p*NAc are α-linked, and a $J_{1,2}$ value of $\sim$8 Hz showed that Gal*p* is β-linked. The β configuration of Rib*f* followed from the C-1 chemical shift of δ 108.3.[14]

Low-field displacements of the NMR signals for C-3 of GlcNAc to δ 80.9, C-4 of Gal to δ 77.6, C-4 of GalA to δ 80.0 and C-2 of Rib to δ 81.5, i.e., by 5.5–9.2 ppm as compared with their positions in the corresponding non-substituted monosaccharides,[14] revealed the sugar substitution pattern. The 2D ROESY spectrum showed the following correlations between the anomeric protons and protons at the linkage carbons: Gal H-1,GlcNAc H-3 at δ 4.48/3.90; GlcNAc H-1,GalA H-4 at δ 4.95/4.45; GalA H-1,Rib H-2 at δ 5.30/4.26; and Rib H-1,Gal H-4 at δ 5.41/4.06. These data confirmed the positions of substitution of the monosaccharides and established their sequence in the repeating unit.

The position of the *O*-acetyl group was determined by comparison of the NMR spectra of the initial and *O*-deacetylated polysaccharide. The most significant changes were observed for the ^{13}C NMR signals of Rib, which were split into two series (with C-1–C-5 at δ 108.6, 81.3, 73.6, 82.7, and 63.4 in the major series and δ 108.3, 81.5, 72.9, 84.2, and 63.8, in the minor series, respectively). In the spectrum ^{1}H NMR of the initial O-polysaccharide, a part of the H-3 signal of Rib was in a lower field than in the spectrum of the *O*-deacetylated polysaccharide (δ 5.15 versus 4.77) due to a deshielding effect of the *O*-acetyl group, which is thus located at position-3 of this monosaccharide. The ratios of the signal intensities for the O-acetylated and non-acetylated Rib*f* residues showed that the degree of acetylation is $\sim$70%.

Based on the data obtained, it was concluded that the O-polysaccharide of *P. mirabilis* O-9 has the structure shown in Fig. 3.

Serological studies with rabbit anti-*P. mirabilis* O-9 serum showed the importance of the *O*-acetyl group in manifesting the immunospecificity of the O-9 antigen. Indeed, while the LPS and the O-polysaccharide inhibited reaction in the homologous system in ELISA at a dose of 9 and 625 ng, respectively, the O-deacetylated polysaccharide was inactive even at a dose 5 mg. A lower reactivity of the O-polysaccharide compared to the LPS could be accounted for by the importance of the core-lipid A moiety of the LPS for the optimal exposure of the polysaccharide chain for antibodies binding to the O-polysaccharide or/and by the presence of antibodies to the LPS core.

In ELISA, the LPS of two strains, *P. vulgaris* O-25 and *Proteus penneri* 14, cross-reacted with anti-*P. mirabilis* O-9 serum at titers 1:51,200 and 1:6400 (the titer for the homologous LPS was 1:1,638,000). In a Western blot, anti-*P. mirabilis* O-9 serum strongly reacted with both slow- and fast-migrating bands of the homologous LPS corresponding to the LPS species with and without polysaccharide chain, respectively, and, although faintly but clearly, recognized slow migrating bands of the LPS of *P. vulgaris* O-25 and *P. penneri* 14. Therefore, the cross-reactive epitopes reside on the O-polysaccharide chains of the LPS. Comparison of the O-polysaccharide structures of the cross-reactive strains[15–17] (Fig. 3) showed that they all contain Rib*f* and Glc*p*NAc and revealed the presence of a common →2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-D-Glc*p*NAc trisaccharide fragment in *P. mirabilis* O-9 and *P. penneri* 14. The O-polysaccharide of *P. vulgaris* O-25 includes a similar trisaccharide fragment in which β-D-Gal*p* is replaced with β-D-Gal*p*NAc. It seems likely that these trisaccharides are involved in epitopes on the O-polysaccharides that are responsible for the cross-reactivity. Sharing of common oligosaccharide fragments in the O-polysaccharides that provide cross-reactivity of strains is rather common in *Proteus*.[11]

3. Experimental

3.1. Bacteria and growth conditions

P. mirabilis O-9, strain PrK 18/57, and *P. vulgaris* O-25, strain PrK 48/57, were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *P. penneri* 14 came from the collection of the Institute of Microbiology and Immunology, University of Lodz. The bacteria were cultivated under aerobic conditions in a fermenter (Chemap AG, Switzerland) in nutrient broth (BTL, Poland) under controlled conditions (37 °C, pH 7.4–7.6, 11 L min^{-1} oxygen). Cells were harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilized. The LPS was isolated by the phenol–water procedure[12] and purified by treatment with DNAse and RNAse (Boehringer Mannheim, Germany) as described.[18] The LPS preparations thus obtained were essentially free of nucleic acid and contained < 2.5% proteins.

Proteus mirabilis O9 (this work)

→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→
3
|
~70% OAc

Proteus vulgaris O25[a]

Sug1*p*
1
↓
3
→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→

Proteus penneri 14[b,c]

→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-β-Sug2*p*-(1→4)-α-Sug3*p*-(1→

Fig. 3. Structures of the O-polysaccharides of the cross-reactive *Proteus* strains. Sug1 stands for 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose, Sug2 for 3-*N*-(*N*-acetyl-D-alanylamino)-3,6-dideoxy-D-glucose, and Sug3 for D-galacturonoyl-L-alanine. Structurally similar trisaccharide fragments are shown in bold type.[a]Ref. 15; [b]Ref. 16; [c]Ref. 17.

3.2. Degradation of the LPS

The LPS of *P. mirabilis* O-9 (134 mg) was hydrolysed with aq 2% HOAc at 100 °C for 2.5 h, and a lipid precipitate was removed by centrifugation at 13,000*g*. The carbohydrate portion was fractionated by GPC on Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a Knauer differential refractometer (Germany) to give a high-molecular-mass polysaccharide (18.5 mg).

3.3. Monosaccharide analysis

The O-polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), and the sugars were identified as their alditol acetates by GLC using a Hewlett–Packard 5989A instrument equipped with an HP-5 column and a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[13] using a Hewlett–Packard 5880 instrument with a DB-5 column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

3.4. Methylation analysis

Methylation of the O-polysaccharide was performed with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide.[19] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in monosaccharide analysis, reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10–10L mass spectrometer (France) using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}. A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h) prior to hydrolysis.

3.5. NMR spectroscopy

The samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.96% D_2O at 30 °C, using internal acetone (δ_H 2.225, δ_C 31.45) as the internal reference. The spectra were recorded on a Bruker DRX-500 MHz spectrometer (Germany) equipped with a SGI INDY computer workstation. 2D spectra were obtained using standard Bruker software, and the XWINNMR 2.1 program (Bruker) was used to acquire and process the NMR data. The parameters used for 2D experiments were essentially the same as described previously.[20] A mixing time of 300 ms was used in 2D TOCSY and ROESY experiments.

3.6. Rabbit anti-O sera

O-Antiserum against *P. mirabilis* O-9 was obtained by immunization of rabbits with heat-inactivated bacteria (100 °C, 2.5 h). To prepare the immunogen, lyophilized bacteria were suspended in saline (aq 0.85% NaCl) at a concentration of 1 mg mL^{-1}. Animals were injected intravenously with 50, 100, 100, 200 and 500 μg of immunogen in 500 μL of saline on days 1, 4, 7, 11 and 16, respectively. Seven days after the last immunization, blood was taken by cardiac puncture, and serum was obtained and stored at −20 °C.

3.7. ELISA and inhibition

NUNC MaxiSorp microtitre U-bottom plates were coated with 50 ng LPS per well in 50 μL NaCl/P_i (15 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) and left at 4 °C overnight. The plates were washed with phosphate-buffered saline, blocked with 2% BSA (Sigma, USA) in NaCl/P_i at 37 °C for 1 h in damp baths and then washed three times with NaCl/P_i. The plates were incubated with rabbit anti-*P. mirabilis* O-9 serum for 1 h at 37 °C and washed four times with NaCl/P_i. Diluted (1:1000) goat *anti*-rabbit IgG conjugated with peroxidase (Sigma, USA) in 1% BSA in NaCl/P_i was used as the second antibody, the plates were washed four times with NaCl/P_i and once with 0.05 M phosphate–citrate buffer pH 5.0 (substrate buffer). A solution of 0.4 mg *o*-phenylenediamine dihydrochloride (Sigma, USA) in 1 mL of substrate buffer and 40 μL of 0.1% H_2O_2 (POCH, Poland) was freshly prepared and added. After 25 min at ambient temperature, the reaction was stopped by adding 0.5 M H_2SO_4, and the absorbance was measured at 492 nm with an EL312e Microplate Reader (BIO-TEK Instruments, USA).

Serial dilutions of an inhibitor (1.5–5000 ng) in 50 μL of PBS were mixed in V-shaped microtitre plates (NUNC) with an equal volume of anti-*P. mirabilis* O-9 serum diluted with PBS to give the absorbance $A_{492} = 1.2$. After incubation at 37 °C for 15 min, the mixture was transferred to *P. mirabilis* O-9 LPS-coated ELISA plates, and the next steps were performed as described above. The last dilution of inhibitor resulting in 50% inhibition was read as the inhibitory value.

3.8. SDS-PAGE and Western blot

SDS-PAGE was performed according to the procedure of Laemmli,[21] using 4% stacking gel and 12.5% resolving gel. The gels were stained with silver nitrate (Sigma, USA) according to Tsai and Frasch[22] or electoblotted onto nitrocellulose sheets (Schleicher & Schuell, Germany) by tank blotting as described.[23] After drying, the nitrocellulose plate was washed for 15 min with blotting buffer (50 mM Tris·HCl, 0.2 M NaCl, pH 7.45) and

blocked for 60 min with blotting buffer containing 10% skimmed milk. The filter paper was incubated for 90 min at room temperature with diluted (1:100) polyclonal anti-*P. mirabilis* O-9 serum in the same buffer and washed (6 × 5 min). The secondary antibodies used in immunoblotting were the same as in ELISA. The color development reagent was 4-chloro-1-naphtol (Sigma, USA) with H_2O_2 as a substrate, and the development reaction was stopped after 10 min by washing with distilled water.

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