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Note

Structure of the O-polysaccharide of *Proteus mirabilis* OC (CCUG 10702) from a new proposed *Proteus* serogroup O75

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Abstract—A neutral O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Proteus mirabilis* OC (CCUG 10702) and studied by sugar and methylation analyses and ^{1}H and ^{13}C NMR spectroscopy. The following structure of the tetrasaccharide repeating unit of the polysaccharide was established:

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

Based on the unique structure of the O-polysaccharide and serological data, we propose classifying *P. mirabilis* OC (CCUG 10702) into a new separate *Proteus* serogroup O75. A weak cross-reaction of O-antiserum against *P. mirabilis* OC with the lipopolysaccharide of *P. mirabilis* O49 was accounted for by a similarity in the O-polysaccharide structures.

Keywords: *Proteus mirabilis*; Lipopolysaccharide; O-Polysaccharide; O-Serogroup; Epitope

Bacteria of the genus *Proteus* are Gram-negative bacilli from the *Enterobacteriaceae* family. They are commonly distributed in the natural environments such as soil and water. *Proteus* rods are also human and animal opportunistic pathogens, which under favourable conditions, may cause various infections. The most important ones are urinary tract infections which often lead to complications such as the formation of kidney stones, chronic pyelonephritis and catheter obstructions.[1,2]

Bacteria of the genus *Proteus* are divided into five species: *Proteus mirabilis*, *Proteus vulgaris*, *Proteus penneri*, *Proteus hauseri* and *Proteus myxofaciens*. To this genus belong also three unnamed *Proteus* genomospecies: 4, 5 and 6.[3,4]

The outer membrane lipopolysaccharide (LPS) is considered to be an important potential virulence factor of these bacteria. The polysaccharide chain of LPS (O-antigen) defines the serological specificity of *Proteus*.

Based on the immunospecificity of LPS, two species: *P. mirabilis* and *P. vulgaris* were classified by Kauffmann and Perch into 49 O-serogroups.[5] However, this classification scheme is not complete. Additional O-serogroups for *P. mirabilis* and *P. vulgaris*[6,7] as well as *P. penneri*[8–10] and *P. myxofaciens*[11] have been proposed.

In this study, we report on the structure of a neutral O-polysaccharide of *P. mirabilis* OC (CCUG 10702) having a tetrasaccharide repeating unit and its serological relatedness to LPS of two other *Proteus* strains.

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Based on the data obtained, we propose classifying this strain into a new *Proteus* serogroup O75.

The polysaccharide was obtained by mild acid degradation of the LPS isolated from *P. mirabilis* OC by the phenol/water procedure.[12] Sugar analysis of the alditol acetates after full acid hydrolysis of the polysaccharide revealed GlcN, GalN, Gal and Rha. GLC analysis of the acetylated glycosides with (*R*)-2-octanol showed that Rha has the L-configuration and the other monosaccharides have the D-configuration.

The ^{13}C NMR spectrum showed that the polysaccharide is regular. It contained signals for four anomeric carbons at δ 100.8–103.6, one CH_3–C group (C-6 of Rha) at δ 17.6, three HOCH_2–C groups (C-6 of GlcN, Gal and GalN) at δ 61.0–61.7, two nitrogen-bearing carbons (C-2 of GlcN and GalN) at δ 56.9 and 53.3, 14 other sugar ring carbons at δ 68.3–83.1 and two *N*-acetyl groups (CH_3 at δ 23.1 and 23.4, CO at δ 175.1 and 175.8). The absence in the ^{13}C NMR spectrum of signals from the region δ 84–88 characteristic for furanosides[13] showed that all monosaccharides are in the pyranose form (Fig. 1).

Accordingly, the ^{1}H NMR spectrum of the polysaccharide contained signals for four anomeric protons in the region at δ 4.66–5.01, one CH_3–C group (H-6 of Rha) at δ 1.31, two *N*-acetyl groups at δ 2.01 and 2.05 and other protons at δ 3.47–4.38.

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-galactose, 2-acetamido-2-deoxy-D-glucose (GlcNAc), 2-acetamido-2-deoxy-D-galactose (GalNAc) and L-rhamnose.

The ^{1}H NMR spectrum of the polysaccharide was assigned using 2D COSY, TOCSY and ROESY experiments (Table 1). The TOCSY spectrum showed cross-peaks of H-1 with H-2 up to 6 for GlcNAc and with H-2 up to 4 for GalNAc and Gal. H-5 and H-6a,6b protons of GalNAc were found by H-1/H-3,5 cross-peaks in the ROESY spectrum and H-5/H-6a,6b cross-peaks in the COSY spectrum. The spin system of Rha was identified by correlations of H-1 with H-2 and H-3 in the TOCSY and H-3 with H-4 up to 6 in the COSY spectrum. The remaining cross-peak in the COSY spectrum was assigned as the correlation H-5/H-6a,6b of Gal residue.

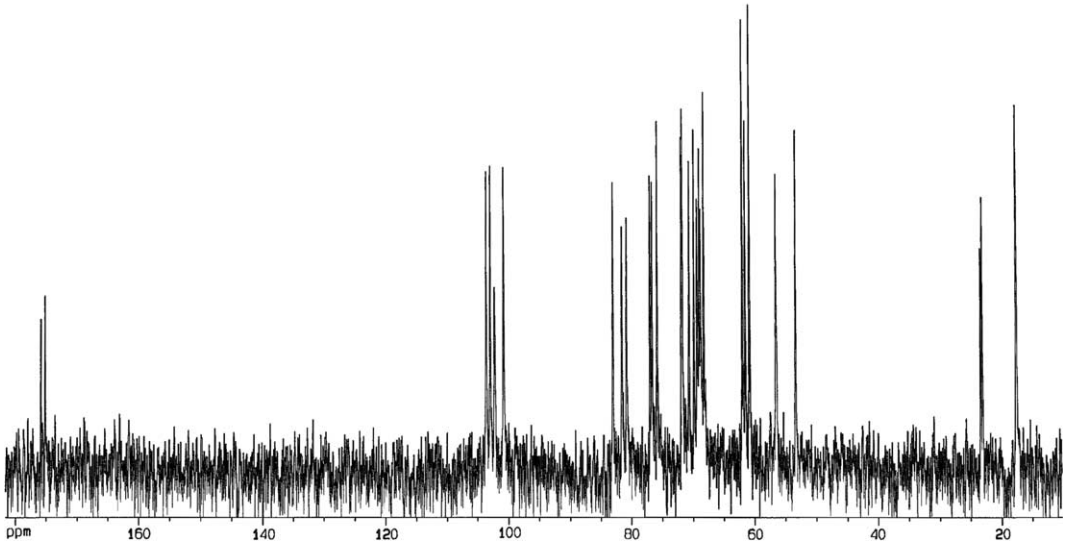


Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* OC.

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

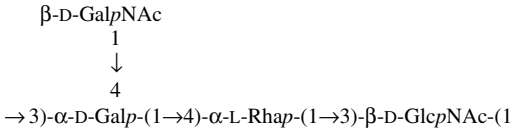
Sugar residue	1	2	3	4	5	6
→3)-β-D-Glc*p*NAc-(1→	4.66	3.92	3.60	3.55	3.47	3.82, 3.93
	103.6	56.9	83.1	69.3	76.7	61.7
→3,4)-α-D-Gal*p*-(1→	5.01	3.74	3.90	4.38	4.25	3.66, 3.80
	100.8	68.3	80.9	77.0	70.7	61.0
β-D-Gal*p*NAc-(1→	4.83	3.91	3.75	3.92	3.64	3.71, 3.78
	102.8	53.3	71.9	68.8	75.9	61.6
→4)-α-L-Rha*p*-(1→	4.86	3.80	3.84	3.49	4.11	1.31
	102.4	72.0	69.8	81.7	69.1	17.6

The chemical shifts for *N*-acetyl groups are δ 2.01 and 2.05 ($^1H_{Me}$); δ 23.1, 23.3 ($^{13}C_{Me}$) and 175.1, 175.8 ($^{13}C_{CO}$).

The ^{13}C NMR spectrum of the polysaccharide was assigned using a H-detected $^{1}H,^{13}C$ experiments. The residues of GlcNAc and GalNAc were demonstrated by correlations of protons at nitrogen-bearing carbons at δ 3.92 and 3.91 to the corresponding carbons at δ 56.9 and 53.3, respectively. Relatively low-field positions of the signals for C-4 of Rha, C-3 of GlcNAc, C-3 and C-4 of Gal at δ 81.7, 83.1, 80.9 and 77.0, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides,[14] demonstrated the glycosylation pattern of the monosaccharides.

The $J_{1,2}$ coupling constant values of ~3 Hz indicated that Gal is α-linked, whereas the $J_{1,2}$ values of ~8 Hz showed that both GlcNAc and GalNAc residues are β-linked. The position of the signals for H-5 at δ 4.05 and C-5 at δ 70.5 indicated that Rha residue is α-linked (compare published data for α- and β-rhamnopyranose).[15]

The ROESY spectrum of the polysaccharide showed GlcNAc H-1/Gal H-3, Gal H-1/Rha H-4, Rha H-1/GlcNAc H-3 and GalNAc H-1/Gal H-4 correlations at δ 4.66/3.90, 5.01/3.49, 4.86/3.60 and 4.83/4.38, respectively. Methylation analysis of the polysaccharide resulted in the identification of 2,6-di-*O*-methylhexose (derived from Gal), 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methyl)acetamidohexose (derived from GlcNAc), 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidohexose (derived from GlcNAc) and 6-deoxy-2,3-di-*O*-methylhexose (derived from Rha). The results of methylation analysis are in agreement with the glycosylation pattern revealed by NMR spectroscopy (see above) and these data defined the sequence of the monosaccharide residues and, therefore, the O-specific polysaccharide of *P. mirabilis* OC has the following structure:



Rabbit polyclonal O-antiserum against *P. mirabilis* OC was tested in an enzyme immunosorbent assay (EIA) with LPS from the complete set of *Proteus* strains representing all *Proteus* O-serogroups, including 39 *P. mirabilis*, 27 *P. vulgaris* and 24 *P. penneri* strains. From them, only two LPS, those from *P. vulgaris* O19 (strain 37/57) and *P. mirabilis* O49 (strain 75/57) were cross-reactive. Whereas the homologous LPS showed a strong reaction (reciprocal titres 51,200 in passive immunohaemolysis and 512,000 in enzyme immunosorbent assay), the cross-reactions were significantly weaker (reciprocal titres 3200 and 4000 for the LPS of *P. vulgaris* O19, 6400 and 4000 for the LPS of *P. mirabilis* O49, respectively). The specificity of the cross-reactions was confirmed by inhibition tests (Table 2). As little as 1 ng of the homologous LPS was sufficient to fully inhibit the reaction in both assays, whereas two other LPS were inactive even in a dose of 1000 ng.

Table 2. Inhibition of PIH and EIA with LPS in the homologous system *P. mirabilis* OC LPS/*P. mirabilis* OC O-antiserum

Inhibitor (LPS)	Minimal inhibitory dose (ng)	
	PIH	EIA
P. mirabilis OC	*1*	*1*
P. mirabilis O49	>1000	>1000
P. vulgaris O19	>1000	>1000

Data for the homologous LPS are italicized.

In Western blot after SDS/PAGE (Fig. 2A), *P. mirabilis* OC O-antiserum recognized both slow- and fast-migrating bands of the homologous LPS and that of *P. mirabilis* O49, which correspond to high- and low-molecular-mass LPS species with and without O-polysaccharide chain, respectively. The O-antiserum bound also fast-migrating bands of the *P. vulgaris* O19 LPS. Absorption of the O-antiserum with the *P. vulgaris*

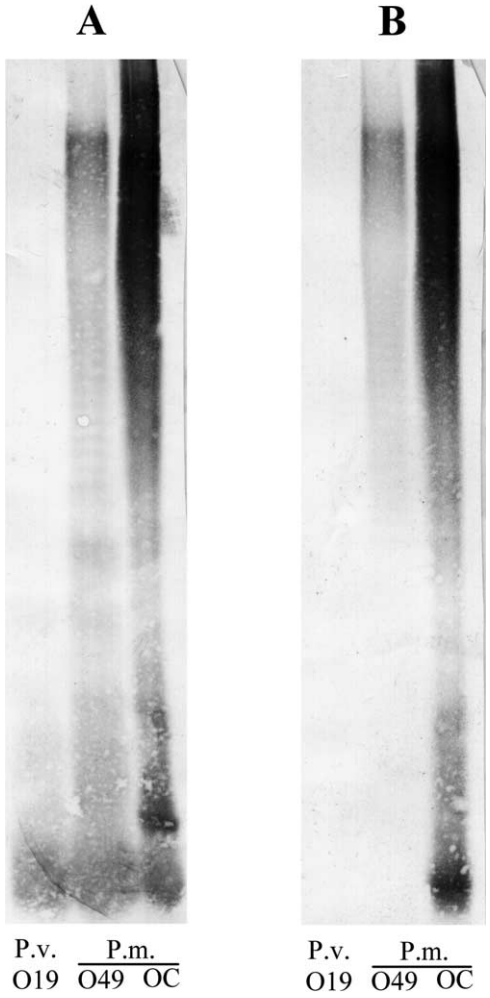


Figure 2. Western blot of *Proteus* lipopolysaccharides with *P. mirabilis* OC O-antiserum (A) and *P. mirabilis* OC O-antiserum absorbed with *P. vulgaris* O19 LPS (B). *P.v.* stands for *P. vulgaris* and *P.m.* for *P. mirabilis*.

Table 3. Reactivity of the absorbed *P. mirabilis* OC O-antiserum with *Proteus* lipopolysaccharides in EIA

Origin of LPS	Reciprocal titre for LPS from		
	P. mirabilis OC	*P. mirabilis* O49	*P. vulgaris* O19
Control	512,000	4000	4000
P. mirabilis OC	*<1000*	*<1000*	*<1000*
P. mirabilis O49	128,000	<1000	<1000
P. vulgaris O19	128,000	1000	<1000

Non-absorbed O-antiserum was used as control. Data for the homologous LPS are italicized.

O19 LPS abolished the reactivity with fast-migrating bands of *P. vulgaris* O19 and *P. mirabilis* O49 (Fig. 2B) and only reduced the reactivity with the LPS of *P. mirabilis* OC.

The reactivity in EIA of *P. mirabilis* OC O-antiserum with all tested antigens was completely abolished when it was absorbed with the homologous LPS (Table 3). Absorption with the *P. mirabilis* O49 LPS removed antibodies against this LPS and that of *P. vulgaris* O19 but only slightly decreased the reactivity with the homologous LPS. Absorption with the *P. vulgaris* O19 LPS abolished the reactivity with this LPS and decreased the reactivity with the LPS of *P. mirabilis* O49 and OC.

The EIA and SDS/PAGE data with the absorbed O-antisera indicate that *P. mirabilis* OC antibodies recognize both O-polysaccharide- and core-associated epitopes on the LPS. The latter is common for all strains studied, whereas the former is shared by *P. mirabilis* OC and O49. Comparison of the chemical structures of the O-polysaccharides from the LPS of *P. mirabilis* OC and O49 (Fig. 3) showed the presence of a common α-L-Rha-(1→3)-β-D-GlcNAc disaccharide fragment, which is the most likely epitope responsible for the cross-reactivity. No conclusion could be made about the nature of the core-associated epitope(s) because the LPS core structures of the strain studied have not been defined.

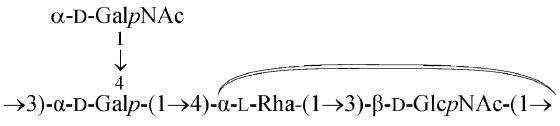
In summary, the cross-reactivity of *P. mirabilis* OC is only weak, and, based on the serological apartness and the unique structure of the O-polysaccharide, we propose classifying *P. mirabilis* OC into a new *Proteus* serogroup O75, in which currently this strain is the single representative.

1. Experimental

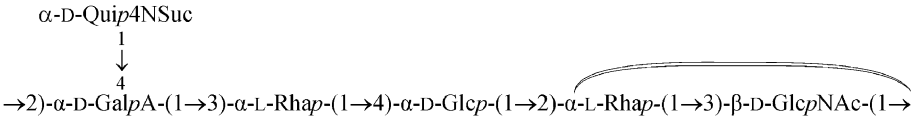
1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

P. mirabilis OC, strain CCUG 10702 was obtained from the Culture Collection of the University of Goeteborg

P. mirabilis OC (this work)



P. mirabilis O49 [16]



P. vulgaris O19 [17]

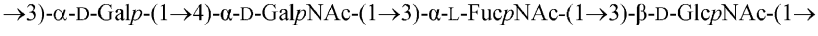


Figure 3. Structure of the O-polysaccharides of *P. mirabilis* OC and O49[16] and *P. vulgaris* O19[17]. Qui4NSuc stands for 4,6-dideoxy-4-(3-carboxypropanoylamino)glucose (4-deoxy-4-succinylaminoquinovose).

(CCUG), Goeteborg, Sweden. Thirty-nine strains of *P. mirabilis* and 27 strains of *P. vulgaris* were from the Czech National Collection of Type Cultures (CNCTC, National Institute of Public Health, Prague, Czech Republic). Twenty-four strains of *P. penneri* were kindly provided by C. M. O'Hara and D. J. Brenner (Centres for Disease Control and Prevention, Atlanta, GA).

The bacteria were cultivated under aerobic conditions on nutrient broth (BTL, Łódź, Poland). The bacterial mass was harvested at the end of the exponential growth phase, centrifuged, washed with water and lyophilized.

Lipopolysaccharide was isolated from dried bacterial cells by hot phenol/water extraction[12] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant.[18]

Delipidation of the LPS (100 mg) was performed with aqueous 2% HOAc at 100 °C until precipitation of lipid. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer (pH 4.5), with monitoring using a Knauer differential refractometer (Germany). The yield of the high molecular-mass O-specific polysaccharide was 23% of the lipopolysaccharide weight.

1.2. Rabbit antiserum and serological assays

Polyclonal O-antiserum was obtained by immunization of rabbits with heat-inactivated bacteria of *P. mirabilis* OC (CCUG 10702) according to the published procedure.[19]

SDS-PAGE (with use of 12% acrylamide), electrotransfer of LPS from gels to nitrocellulose sheets, immunostaining and absorption experiments, enzyme immunosorbent assay (EIA) using LPS and passive immunohaemolysis test (PIH) using alkali-treated LPS as antigen, as well as inhibition experiments were carried out as described.[20] Passive immunohaemolysis was performed with use of increasing amounts (2–200 μg) of LPS.

1.3. Sugar analysis

The polysaccharide was hydrolyzed with 2 M CF_3COOH (120 °C, 2 h), monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (20 °C, 2 h), acetylated with a 1:1 (v/v) mixture of pyridine and Ac_2O (120 °C, 1 h) and analyzed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*R*)-2-octyl glycosides according to the published method.[21,22] GLC was performed using a Hewlett-Packard 5890 instrument equipped with a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min.

1.4. Methylation analysis

Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[23] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, converted into alditol acetates, and analyzed by GLC–MS using the same chromatographic conditions as in GLC.[24]

1.5. NMR spectroscopy

NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D_2O at 30 °C for the polysaccharide, using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 2.1) was used to acquire and process the NMR data. A mixing time of 200 ms was used in 2D TOCSY and ROESY experiments.

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