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Note

Structure of the O-polysaccharide of *Proteus mirabilis* CCUG 10705 (OF) containing an amide of D-galacturonic acid with L-alanine

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Abstract—The structure of the O-polysaccharide of *Proteus mirabilis* CCUG 10705 (OF) was determined by chemical analyses along with one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy. The polysaccharide was found to contain an amide of D-galacturonic acid with L-alanine and based on the uniqueness of the O-polysaccharide structure and serological data, it was suggested to classify *P. mirabilis* OF into a new separate *Proteus* serogroup, O74. A weak cross-reactivity of *P. mirabilis* OF and *P. mirabilis* O5 was observed and accounted for by a similarity of their O-repeating units. The following structure of the polysaccharide of *P. mirabilis* OF was established:

α-D-Glc*p*NAc
1
↓
4
→3)-α-D-Gal*p*A2Ac-(1→3)-α-D-Gal*p*A6(L-Ala)-(1→3)-α-D-Glc*p*NAc-(1→

Keywords: Proteus mirabilis; O-antigen; Lipopolysaccharide; Polysaccharide structure; *N*-(D-Galacturonoyl)-L-alanine

Lipopolysaccharide (LPS) is the major constituent of the outer membrane of the cell wall of Gram-negative bacteria and is considered as an important microbial virulence factor.[1,2] The genus *Proteus* is one of the representatives of Gram-negative bacteria of the family Enterobacteriaceae and currently consists of five named species *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, and three unnamed genomospecies 4, 5 and 6.[3,4]

The polysaccharide chain of LPS (O-antigen, OPS) defines the immunospecificity of Gram-negative bacteria, including *Proteus*. Based on the O-antigens, strains of the genus *Proteus* have been divided into a number of O-serogroups.[5–11] Now, we report on a new structure of the OPS from a formerly non-classified strain, *P. mirabilis* CCUG 10705 (OF), and suggest classifying this strain into a separate *Proteus* serogroup, O74.

LPS was isolated from dried bacterial cells of *P. mirabilis* CCUG 10705 (OF) by the phenol–water extraction[12] and degraded with dilute acetic acid to give a high-molecular-mass OPS. Methanolysis of the OPS followed by GLC analysis of the acetylated methyl glycosides showed the presence of galacturonic acid (GalA) and glucosamine (GlcN), as well as another component, which was identified later as alanine. GLC analysis of the acetylated glycosides with (*S*)-2-butanol showed that both monosaccharides have the D configuration. The L configuration of alanine was determined by GLC of the acetylated (*S*)-2-butyl ester.

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The ^{13}C NMR spectrum of the OPS demonstrated an irregularity due to non-stoichiometric O-acetylation (a characteristic signal for an *O*-acetyl group was observed at δ 21.7). O-Deacetylation resulted in a regular polysaccharide (PS), whose ^{13}C NMR spectrum (Fig. 1) showed signals for four anomeric carbons at δ 96.7–102.3, two CH_2OH groups at δ 61.8 and 63.0 (C-6 of two GlcN residues), a C–CH_3 group at δ 18.0 (C-3 of Ala), three carboxyl (or carboxamide) groups at δ 171.2, 174.1 and 176.9 (C-6 of two GalA residues and C-1 of Ala), three nitrogen-bearing carbons at δ 55.9, 52.9 and 49.9 (C-2 of GlcN and Ala), 14 sugar-ring oxygen-bearing carbons in the region δ 67.9–83.1, and two *N*-acetyl groups (C–CH_3 at δ 23.3 and 23.5, CO at δ 175.1 and 176.0). Accordingly, the ^{1}H NMR spectrum of the PS contained, inter alia, signals for four anomeric protons at δ 5.02–5.36, a CH_3 group at δ 1.45 (3H, H-3 of Ala) and two *N*-acetyl groups at δ 1.96 and 2.05 (3H each). Therefore, the PS has a tetrasaccharide repeating unit containing two residues each of D-GalA (GalA^{I} and GalA^{II}) and D-GlcNAc (GlcNAc^{I} and GlcNAc^{II}), and one residue of L-Ala.

The ^{1}H and ^{13}C NMR spectra of the PS were assigned using 2D COSY, TOCSY, H-detected ^{1}H,^{13}C HSQC, and HMBC experiments (Table 1). Two spin-systems included resonances from H-1 to H-6 with relatively large coupling constants between vicinal protons, thus showing the presence of two monosaccharides with the *gluco* configuration (GlcNAc^{I} and GlcNAc^{II}). They were confirmed by a correlation of the protons at the nitrogen-bearing carbons (H-2) to the corresponding carbons (C-2) at δ 3.83/55.9 and δ 4.13/52.9, which were shown by a ^{1}H,^{13}C HMQC experiment.

The TOCSY spectrum revealed connectivities from H-1 to H-5 for GalA^{II} and from H-1 to H-4 for GalA^{I}; the remaining resonance, showing a cross-peak at δ 4.76/72.4 in the ^{1}H,^{13}C HMQC spectrum, belonged thus to H-5 of GalA^{I}. The C-6 resonances of GalA^{I} and GalA^{II} as well as CO signals of Ala and *N*-acetyl groups were assigned using an ^{1}H,^{13}C HMBC experiment. The HMBC spectrum showed cross-peaks between atoms separated by two and three bonds at δ 176.9/4.47, 176.9/1.45; 174.1/4.76 and 171.2/4.29, which were inter-

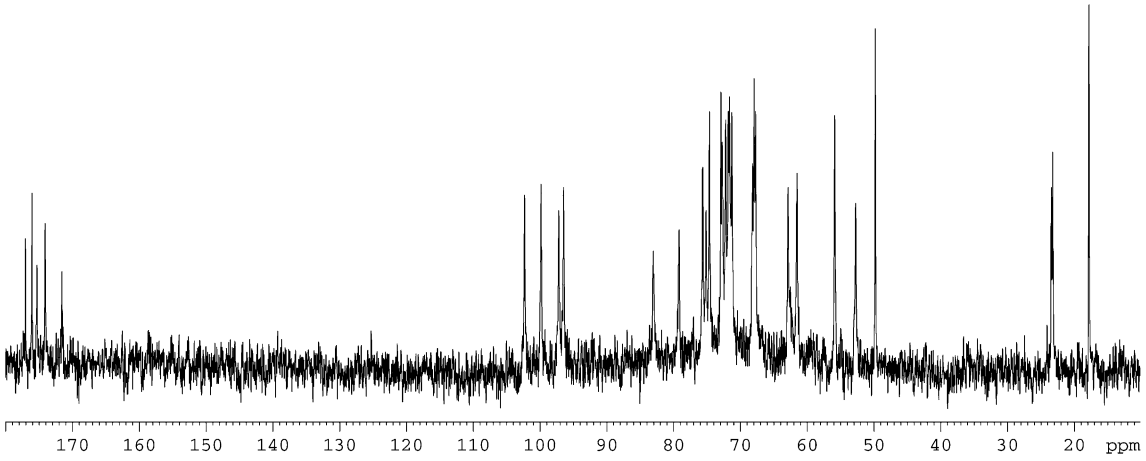


Figure 1. ^{13}C NMR spectrum of the O-deacetylated polysaccharide (PS) of *P. mirabilis* CCUG 10705 (OF).

Table 1. ^{1}H and ^{13}C NMR data of the O-deacetylated polysaccharide of *P. mirabilis* OF (δ, ppm)

Sugar residue		Atom number							
		1	2	3	4	5	6 (6a,6b)	Me	CO
α-D-Glc*p*NAc^{I}-(1→	^{1}H	5.02	3.83	3.77	3.47	3.92	3.85, 3.99	2.05	
	^{13}C	99.8	55.9	71.7	72.0	74.8	63.0	23.5	176.0
→3,4)-α-D-Gal*p*A^{I}-(1→	^{1}H	5.30	4.13	4.13	4.56	4.76	—		
	^{13}C	97.3	67.9	75.5	79.3	72.4	174.1		
→3)-α-D-Gal*p*A^{II}6(L-Ala)-(1→	^{1}H	5.36	4.00	4.06	4.46	4.29	—		
	^{13}C	102.3	68.1	75.9	68.2	73.0	171.2		
→3)-α-D-Glc*p*NAc^{II}-(1→	^{1}H	5.09	4.13	3.93	3.72	4.04	3.74, 3.85	1.96	
	^{13}C	96.7	52.9	83.1	71.8	72.9	61.8	23.3	175.1
L-Ala	^{1}H	—	4.47	1.45					
	^{13}C	176.9	49.9	18.0					

preted as Ala C-1,Ala H-2; Ala C-1,Ala H-3; GalA^{I} C-6,GalA^{I} H-5 and GalA^{II} C-6,GalA^{II} H-5 correlations.

As judged by relatively small $^{3}J_{1,2}$ coupling constants values of ~3 Hz determined from the ^{1}H NMR spectrum for the H-1 signals at δ 5.02–5.36, all sugar residues are α-linked. This conclusion was confirmed by a ROESY experiment, which showed intraresidue H-1,H-2 and H-1,H-3 correlations that are typical of α-linked pyranosides for all sugar residues. Significant downfield displacements of the signals for C-3 and C-4 of GalA^{I}, C-3 of GalA^{II} and C-3 of GlcNAc^{II} to δ 75.5–83.1, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides at δ 70.3–71.7,[13] revealed the glycosylation pattern in the repeating unit.

In addition to the intraresidue correlations, the ROESY spectrum showed a number of strong interresidue cross-peaks at δ 5.02/4.56, 5.30/4.06, 5.36/3.93 and 5.09/4.13, which were assigned to correlations between the following anomeric protons and protons at the linkage carbons: GlcNAc^{I} H-1,GalA^{I} H-4; GalA^{I} H-1, GalA^{II} H-3; GalA^{II} H-1,GlcNAc^{II} H-3; GlcNAc^{II} H-1,GalA^{I} H-3, respectively. These data defined the monosaccharide sequence in the repeating unit.

The position of Ala was determined from a ^{1}H,^{1}H ROESY experiment performed in an H_2O/D_2O mixture (9:1), which revealed correlations for NH protons (Fig. 2). Particularly, the spectrum showed a cross-peak between NH of Ala and H-5 of GalA^{II} at δ 8.05/4.29, which demonstrated the attachment of Ala to the carboxyl group of GalA^{II} via an amide linkage. This conclusion was confirmed by a relatively upfield position of the C-6 signal of GalA^{II} at δ 171.2 that is typical of a carboxamide group. Moreover, the ROESY spectrum showed correlations of NH protons of GlcNAc^{I} and Glc*p*NAc^{II} with the methyl groups of the *N*-acetyl groups at δ_H 7.38/2.05 and 8.23/1.96, respectively, thus confirming N-acetylation of GlcNAc^{I} and GlcNAc^{II}. An interresidue cross-peak between NH of GlcNAc^{II} and H-5 of GalA^{II} observed at δ 8.23/4.29 was predicted by MM3 molecular modelling and confirmed the same absolute configuration of these sugar residues.

Position of the *O*-acetyl group was determined by the analysis of the ^{13}C NMR spectrum of the OPS. As compared with the ^{13}C spectrum of the PS, this showed a displacement of about 60% of the GalA^{I} C-2 signal from δ 67.9 to 70.2. This displacement was due to a deshielding effect of the *O*-acetyl group and indicated partial O-acetylation of GalA^{I} at position 2. This finding was

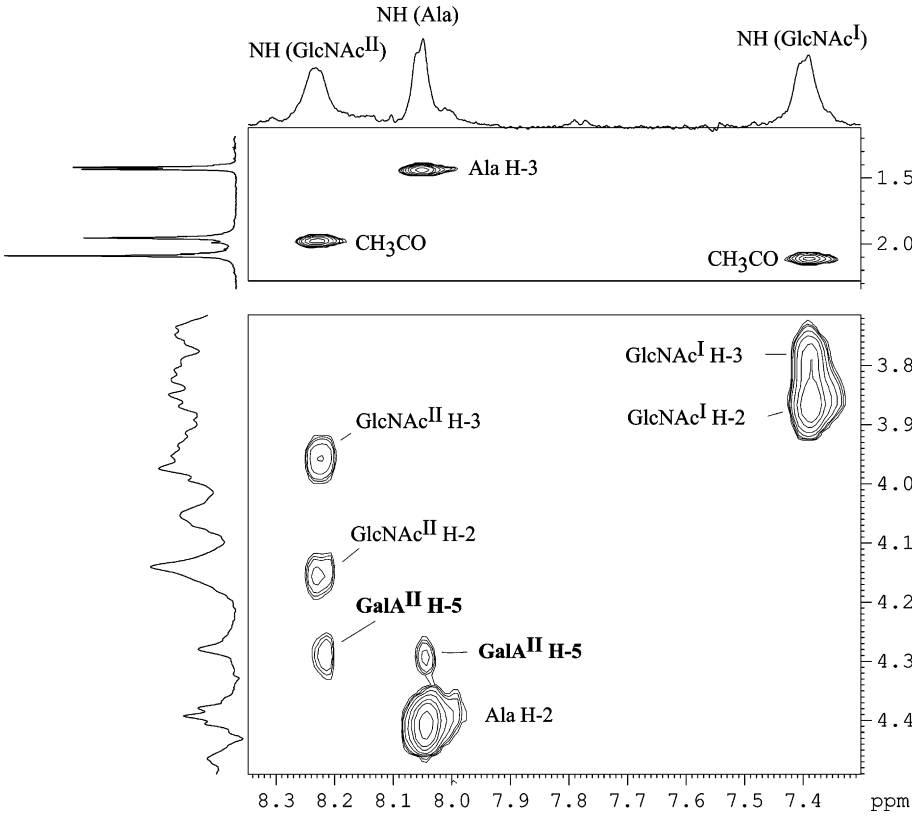


Figure 2. Part of a ^{1}H,^{1}H ROESY spectrum of the O-deacetylated polysaccharide (PS) of *P. mirabilis* CCUG 10705 (OF). The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. Arabic numerals refer to protons in sugar residues and NH denotes protons of amino groups of the corresponding amino sugars and alanine. Interresidue correlations are shown in bold type.

confirmed by an upfield shift by 1.7 ppm of the GalA^{I} C-1 signal, which was caused by a β-effect of O-acetylation.[14] The data obtained allowed establishing the structure of the OPS of *P. mirabilis* CCUG 10705 (OF) shown in Figure 3. An unusual component of the OPS is *N*-(D-galacturonoyl)-L-alanine, which was found earlier in several other *Proteus* OPS's, including those of *P. mirabilis* O27[15] and *P. penneri* 14 (O59).[16]

LPS from 92 strains representing all known *Proteus* serogroups were tested in passive immunohemolysis (PIH) and enzyme immunosorbent assay (EIA) with polyclonal rabbit O-antiserum against *P. mirabilis* CCUG 10705 (OF). Cross-reactions were observed only for LPS of *P. mirabilis* 12/57 and *P. mirabilis* 13/57, which both represent *Proteus* O5 serogroup (Table 2). In Western blot, O-antiserum recognized high-molecular-mass LPS bands *P. mirabilis* 12/57 and *P. mirabilis* 13/57 (data not shown) and, therefore, the common epitope that is responsible for the cross-reactivity, is located on the OPS's.

Comparison of the OPS structures of *P. mirabilis* CCUG 10705 (OF) (this work) and *P. mirabilis* O5[17] showed that they have tetrasaccharide repeating units with the same sugar composition but different structures. In particular, the *P. mirabilis* CCUG 10705 (OF) O-polysaccharide is branched and contains alanine, whereas the *P. mirabilis* O5 OPS is linear and is devoid of alanine. However, both polysaccharides could contain similar terminal tetrasaccharide units if the first monosaccharide of the repeating units is 3-substituted GlcNAc, as found recently in the OPS's of a number of Gram-negative bacteria (e.g., see Ref. 18). Having the same glycosylation pattern and the same monosaccharide sequence, these tetrasaccharide units shown in Figure 3 differ in the configuration of the glycosidic linkage of one of the GlcNAc residues, the position of the *O*-acetyl groups, and the presence or absence of Ala. These differences as well as data of inhibition tests (Table 2) and tests with O-antiserum absorbed with the cross-reactive LPS (data not shown) indicated that *P. mirabilis* CCUG 10705 (OF) and *P. mirabilis* O5 are distantly related. Based on these data, we propose to classify the strain studied into a new, separate *Proteus* serogroup, O74. Formerly, the O74 serogroup had been proposed for another non-classified strain, *P. mirabilis* CCUG 10701 (OB),[19] which was reclassified recently into the existing *Proteus* O23 serogroup.[20]

Table 2. Reactivity of *Proteus* LPS with O-antiserum against *P. mirabilis* CCUG 10705 (OF)

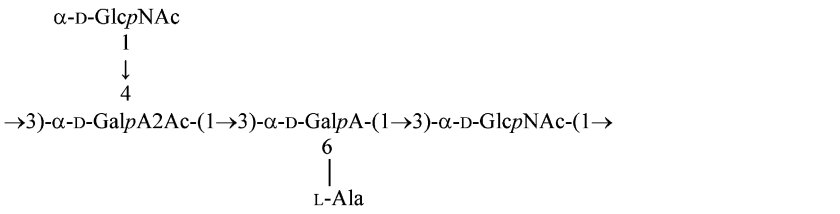
LPS from *P. mirabilis*	Reciprocal titre		Minimal inhibiting dose (ng)	
	PIH	EIA	PIH	EIA
CCUG 10705 (OF)	51,200	12,800	1	4
12/57 (O5)	6400	4000	>1000	>1000
13/57 (O5)	3200	4000	>1000	>1000

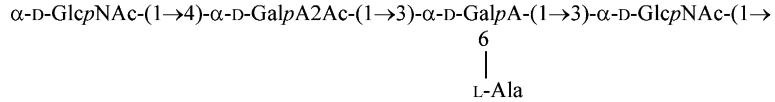
1. Experimental

1.1. Bacterial strain and growth

P. mirabilis OF, strain CCUG 10705 was obtained from the Culture Collection of the University of Goeteborg, Goeteborg, Sweden. The strains of *P. vulgaris* (27) and

P. mirabilis CCUG 10705 (OF)





P. mirabilis O5

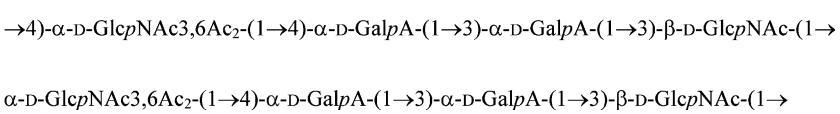


Figure 3. Structures of the O-polysaccharides repeating units and proposed terminal repeating units of the cross-reactive LPS of *P. mirabilis* CCUG 10705 (OF) and *P. mirabilis* O5.

P. mirabilis (39) were from the Czech National Collection of Type Cultures (CNCTC, National Institute of Public Health) Prague, Czech Republic. *P. myxofaciens* strain (CCUG 18769) was provided by E. Falsen (Cultures Collection, University of Goeteborg, Goeteborg, Sweden). Twenty four strains of *P. penneri* as well as *P. hauseri* strain were kindly provided by C. M. O'Hara and D. J. Brenner (Centres for Diseases Control and Prevention, Atlanta, Georgia, USA). The bacteria were grown under aerobic conditions on nutrient broth (BTL, Łódź, Poland). Dry bacterial cells were obtained from aerated liquid culture as described.[21]

1.2. Isolation and degradation of the lipopolysaccharide

The LPS was isolated from dried bacterial cells by extraction with hot aqueous phenol[12] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant.[22] Delipidation of the lipopolysaccharide (117 mg) was performed with aqueous 2% HOAc at 100 °C, until lipid A had been precipitated. 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 a high-molecular-mass OPS was 22% of the LPS weight.

1.3. O-Deacetylation

The OPS (26 mg) was treated with aq 12.5% ammonia at 37 °C for 16 h, the solution was desalted on a column (90 × 2.5 cm) of TSK HW-40 (S) (Merck, Germany) in water and freeze-dried to give the PS (21.1 mg).

1.4. Rabbit antiserum and serological assays

Polyclonal O-antiserum against *P. mirabilis* CCUG 10705 (OF) was obtained by immunization of rabbits with heat-inactivated bacteria according to a published procedure.[23] Enzyme immunosorbent assay (EIA) and passive immunohemolysis (PIH) and absorption experiments were carried out as described. SDS/PAGE (with use of 12% acrylamide) and electrotransfer of LPS from gel to nitrocellulose sheets as well as immunostaining were performed according to a published procedure.[24]

1.5. Sugar analysis

Methanolysis of the PS (1 mg) was carried out using 1 M HCl–MeOH (85 °C, 16 h), followed by acetylation with Ac_2O in pyridine (120 °C, 30 min), and subsequently analyzed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to published methods.[25,26] Absolute configuration of alanine was determined by GLC of acetylated (*S*)-(+)-2-butyl ester using authentic L-alanine and D-alanine, as references. GLC was performed using a Hewlett–Packard 5890 Series II instrument equipped with an HP fused silica column (0.25 mm × 30 m) using a temperature program of 170–180 °C (1 °C min^{-1}) followed by 180–230 °C (7 °C min^{-1}).

1.6. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 60 °C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. 2D NMR experiments were performed using standard Bruker software. A mixing time of 200 and 300 ms was used in TOCSY and ROESY experiments, respectively.

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