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Note

Structure of the O-polysaccharide of *Proteus mirabilis* CCUG 10701 (OB) classified into a new *Proteus* serogroup, O74

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Abstract—An acidic O-polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Proteus mirabilis* CCUG 10701 (OB) and studied by chemical analyses and ^{1}H and ^{13}C NMR spectroscopy. The following structure of the tetrasaccharide repeating unit of the polysaccharide was established:

→ 3)-β-D-Glc*p*NAc6Ac-(1 → 2)-β-D-Gal*p*A4Ac-(1 → 3)-α-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*A-(1 →,

where the degree of O-acetylation at position 6 of GlcNAc is ~50% and at position 4 of β-GalA ~60%. Based on the unique structure of the O-polysaccharide and serological data, it is proposed to classify *P. mirabilis* CCUG 10701 (OB) into a new *Proteus* serogroup, O74.

Keywords: *Proteus mirabilis*; Lipopolysaccharide; O-Specific polysaccharide; Serological classification

Bacteria of the genus *Proteus* are widely distributed in nature and are important human opportunistic pathogens. They cause wound and urinary tract infections, which can result in severe complications, such as acute or chronic pyelonephritis, catheter obstruction, bacteriemia and formation of bladder and kidney stones.[1] Currently, *Proteus* rods are subdivided into five named species, including *Proteus mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, and three unnamed genomospecies 4–6.[2,3]

Based on the serospecificity of the lipopolysaccharide (O-antigen), strains of *P. mirabilis* and *P. vulgaris* are divided into 49 O-serogroups,[4] and later 11 additional O-serogroups have been proposed.[5] Recently, the classification scheme of *Proteus* has been extended by creation of new serogroups, including strains of *P. penneri* [6–10] and *P. myxofaciens*[11] with structurally defined O-antigens. Now we report chemical and serological studies on the O-antigen of *P. mirabilis* CCUG 10701 (OB), which showed the expediency of classification of this strain into a new *Proteus* serogroup.

The polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from *P. mirabilis* CCUG 10701 (OB) by the phenol–water procedure.[12] Sugar analysis of the alditol acetates after full acid hydrolysis of the polysaccharide revealed GlcN and GalN. Methanolysis of the polysaccharide, followed by GLC analysis of the acetylated methyl glycosides, showed the presence of galacturonic acid (GalA). GLC analysis of the acetylated glycosides with (*S*)-2-butanol showed that all sugar residues have the D configuration.

The ^{13}C NMR spectrum of the polysaccharide contained signals having different intensities owing to non-stoichiometric O-acetylation (there were two signals for CH_3 of *O*-acetyl groups at δ 21.4 and 21.6). The ^{1}H NMR spectrum of the polysaccharide included signals for *O*-acetyl and *N*-acetyl groups at δ 1.97–2.13.

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The ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide (Fig. 1) was typical for a regular polymer and showed signals for four anomeric carbons at δ 100.0–103.5, two HO*C*H_2–C groups (C-6 of GlcN and GalN) at δ 61.9 and 61.6, two nitrogen-bearing carbons (C-2 of GlcN and GalN) at δ 55.7 and 49.6, 14 other sugar ring carbons at δ 69.3–83.7, two COOH groups (C-6 of GalA) at δ 173.4 and 173.6 and two *N*-acetyl groups (CH_3 at δ 23.3 and 24.1, CO at δ 175.2 and 175.8). The absence in the ^{13}C NMR spectrum of signals in the region δ 84–88, that are characteristic for furanosides, showed that all monosaccharides are in the pyranose form. The ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide contained signals for four anomeric protons in the region at δ 4.57–5.38, two *N*-acetyl groups at δ 1.98 and 2.10 and other protons at δ 3.38–4.42.

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose and two residues of D-galacturonic acid.

Methylation analysis of the *O*-deacetylated polysaccharide resulted in identification of 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose. When the methylated polysaccharide was carboxyl reduced prior to hydrolysis, 2,3-di-*O*-methylgalactose and 3,4-di-*O*-methylgalactose were identified in addition to the sugars mentioned above, both evidently deriving from GalA. Therefore, the polysaccharide is linear and contains 3-substituted residues of GlcNAc and GalNAc and 2-substituted and 4-substituted residues of GalA.

The ^{1}H and ^{13}C NMR spectra of the *O*-deacetylated polysaccharide were assigned using 2D COSY, TOCSY, ROESY and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). $J_{1,2}$ coupling constant values of ~3 Hz indicated that GalNAc and one of the GalA residues are α-linked, whereas the $J_{1,2}$ values of ~8 Hz showed that GlcNAc and the other GalA residue are β-linked. The TOCSY spectrum showed cross-peaks of H-1 with H-2–H-6 of GlcNAc, H-2–H-5 of GalNAc and H-2–H-4 of two GalA residues. The remaining proton of β-GalA (H-5) was found by a H-4,H-5 correlation in the COSY spectrum and a H-1,H-5 correlation in the ROESY spectrum, and those of GalNAc were revealed by H-4,H-5 and H-5,H-6a,6b correlations in the COSY spectrum. The position of H-5 of α-GalA was defined by the H-5,C-5 correlation in the ^{1}H,^{13}C HMQC spectrum, which was the only cross-peak that remained unassigned after the assignment of all other signals having been performed. The residues of GlcNAc and GalNAc were demonstrated by correlations in the HMQC spectrum of

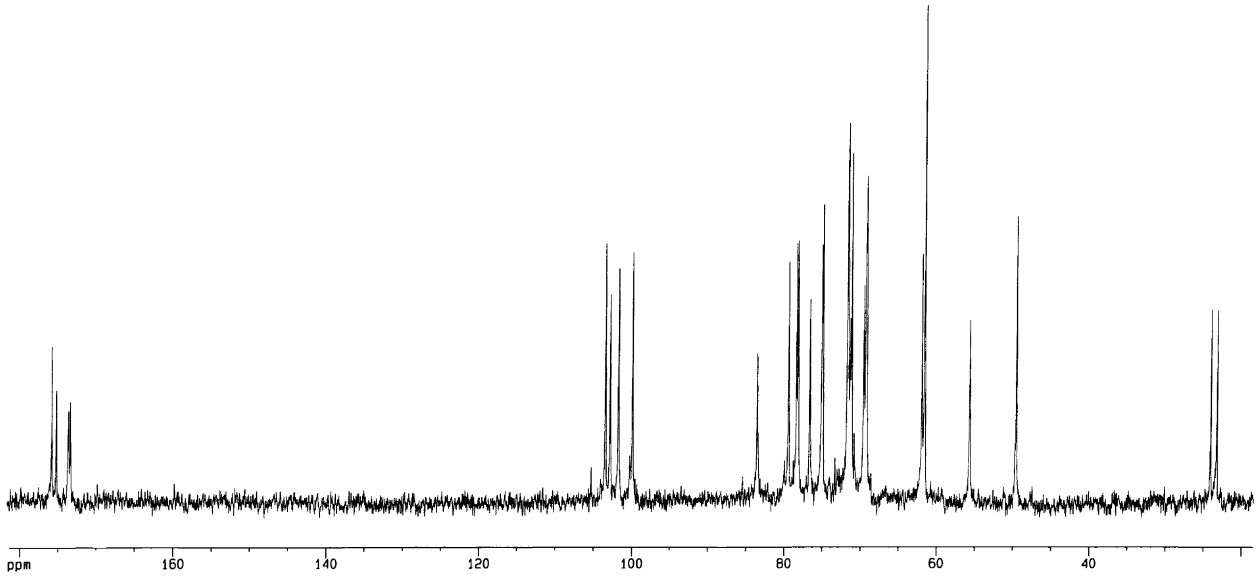

Figure 1. ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide of *P. mirabilis* CCUG 10701 (OB).

Table 1. ^{13}C NMR data (δ, ppm) for the *O*-deacetylated polysaccharide of *P. mirabilis* CCUG 10701 (OB)[a]

Residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Glc*p*NAc-(1→	102.9	55.7	83.7	71.9	76.7	61.9
→2)-β-D-Gal*p*A-(1→	103.5	78.3	75.0	71.3	75.2	173.4
→3)-α-D-Gal*p*NAc-(1→	100.0	49.6	78.5	69.3	71.7	61.6
→4)-α-D-Gal*p*A-(1→	101.8	69.5	69.7	79.5	71.3	173.6

[a]Chemical shifts for NAc are δ 23.3, 24.1 (both Me), 175.2 and 175.8 (both CO).

Table 2. ^{1}H NMR data (δ, ppm) for the *O*-deacetylated polysaccharide of *P. mirabilis* CCUG 10701 (OB)[a]

Residue	H-1	H-2	H-3	H-4	H-5	H-6a,6b
→3)-β-D-Glc*p*NAc-(1→	4.83	3.76	3.73	3.65	3.38	3.71, 3.89
→2)-β-D-Gal*p*A-(1→	4.57	3.70	3.72	4.17	4.27	
→3)-α-D-Gal*p*NAc-(1→	4.95	4.30	4.00	4.37	4.36	3.73
→4)-α-D-Gal*p*A-(1→	5.38	3.90	3.98	4.41	4.42	

[a]Chemical shifts for NAc are δ 1.98 and 2.10.

the protons at the nitrogen-bearing carbons to the corresponding carbons at δ 3.76/55.7 and 4.30/49.6, respectively.

Relatively low-field positions of the signals for C-2 of β-GalA, C-3 of GlcNAc and GalNAc and C-4 of α-GalA at δ 83.7, 78.5, 78.3 and 79.5, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides, demonstrated the glycosylation pattern in the repeating unit.[13] In addition to the intraresidue cross-peaks, the ROESY spectrum showed cross-peaks between the following *trans*-glycosidic protons: GlcNAc H-1, β-GalA H-2, β-GalA H-1, GalNAc H-3, GalNAc H-1, α-GalA H-4 and α-GalA,GlcNAc H-3 at δ 4.83/3.70, 4.57/4.00, 4.95/4.41 and 5.38/3.73, respectively. These data were in agreement with the glycosylation pattern and defined the sequence of the monosaccharide residues in the repeating unit. Therefore, the *O*-deacetylated polysaccharide of *P. mirabilis* CCUG 10701 (OB) has the following structure:

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

Positions of the *O*-acetyl groups were determined by a ^{1}H,^{13}C HMQC experiment with the initial O-polysaccharide. As compared with the HMQC spectrum of the *O*-deacetylated polysaccharide, this showed a displacement of about half of the GlcNAc H-6,C-6 and β-GalA H4,C-4 of cross-peaks from δ 3.71/61.9 (6a) and 3.89/61.9 (6b) to 4.29/65.0 and 4.43/65.0 and from δ 4.17/71.3 to 5.49/73.8, respectively. The displacement was due to a deshielding effect of the *O*-acetyl group and indicated partial O-acetylation of GlcNAc at position 6 and β-GalA at position 4. The degree of O-acetylation at these positions was estimated based on the signal intensities as ~50% and ~60%, respectively. The O-acetylation pattern was confirmed by upfield shifts (β-effects of O-acetylation[14]) of the signal for C-5 of GlcNAc by 1.7 ppm and those for C-3 and C-5 of β-GalA by 0.8 and 2.5 ppm, respectively. On the basis of the data obtained, it was concluded that the O-polysaccharide of *P. mirabilis* CCUG 10701 has the following structure:

→ 3)-β-D-Glc*p*NAc6Ac-(1 → 2)-β-D-Gal*p*A4Ac-(1
→ 3)-α-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*A-(1 →,

where O-acetylation at both positions is nonstoichiometric.

No serological cross-reactivity was observed between rabbit polyclonal O-antiserum against *P. mirabilis* CCUG 10701 (OB) and lipopolysaccharides from the full set of serologically distinct *Proteus* strains in passive immunohemolysis and enzyme immunosorbent assays. This finding is in accordance with the structure of the O-antigen *P. mirabilis* CCUG 10701 (OB), which is unique among the known *Proteus* O-polysaccharide structures. Therefore, we propose for the strain studied a new *Proteus* serogroup, O74, which consists of the single strain *P. mirabilis* CCUG 10701 (OB).

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

P. mirabilis OB, strain 10701, was kindly provided by Dr. Enevald Falsen from the Culture Collection of University of Goeteborg (CCUG). The bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Poland). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water and lyophilised as described.[15]

Lipopolysaccharide was isolated from dried bacterial cells by hot phenol–water extraction[12] and purified with trichloroacetic acid.[16] Delipidation of the lipopolysaccharide (120 mg) was performed with aq 2% HOAc at 100 °C during 3 h until the precipitation of lipid A was complete. 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-polysaccharide was 23% of the lipopolysaccharide weight.

The O-polysaccharide (30 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) (E. Merck, Germany) in water and freeze-dried to give an *O*-deacetylated polysaccharide (19 mg).

1.2. Sugar analysis

The O-polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (20 °C, 2 h),

acetylated with 1:1 (v/v) pyridine–Ac_2O (120 °C, 1 h) and analysed by GLC. Methanolysis of the polysaccharide (1 mg) was carried out using 1 M HCl in MeOH (85 °C, 16 h), followed by acetylation with Ac_2O in pyridine (120 °C, 30 min), and subsequent GLC analysis. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-butyl glycosides as previously described.[17,18] GLC was performed on a Hewlett–Packard 5890 Series II instrument equipped with an HP-1 fused silica column (0.25 mm × 30 m) using a temperature program from 170 to 180 °C at 1 °C min^{-1}, and then from 180 to 230 °C at 7 °C min^{-1}.

1.3. Methylation analysis

Methylation of the polysaccharide (2 mg) was performed with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide.[19] A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in the sugar analysis, converted into the alditol acetates and analysed by GLC–MS on a TermoQuest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1}.

1.4. NMR spectroscopy

Prior to measurements, samples were freeze-dried twice from D_2O. NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D_2O at 30 °C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 2.6) was used to acquire and process the NMR data. A mixing time of 200 and 30 ms was used in TOCSY and ROESY experiments, respectively.

1.5. Rabbit antiserum and serological assays

Polyclonal, O-antiserum was obtained by immunisation of rabbits with heat-inactivated bacteria of *P. mirabilis* CCUG 10701 (OB) according to published procedure.[20] Passive immunohemolysis and enzyme immunosorbent assay were performed as described earlier.[21]

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