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Structure of the O-specific polysaccharide of *Proteus mirabilis* O11, another *Proteus* O-antigen containing an amide of D-galacturonic acid with L-threonine

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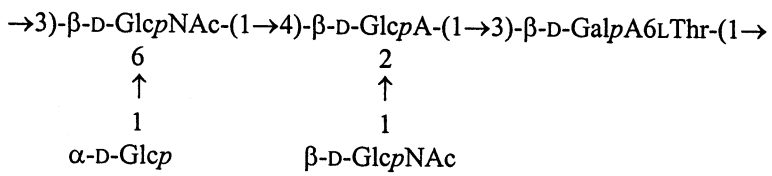
Abstract

The O-specific polysaccharide of *Proteus mirabilis* O11 was studied by sugar analysis, Smith degradation, ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional COSY, TOCSY, NOESY, and ^{1}H-detected $^{1}H,^{13}C$ HMQC experiments. The following structure of a pentasaccharide repeating unit of the polysaccharide was established:



where D-GalA6LThr is *N*-(D-galacturonoyl)-L-threonine. ELISA with anti-*P. mirabilis* O11 serum showed that D-GalA6LThr is of minor importance for manifesting the O11 immunospecificity.

Keywords: *Proteus mirabilis*; D-Galacturonoyl-L-threonine; O-Antigen; O-Specific polysaccharide; Lipopolysaccharide; Structure

1. Introduction

Proteus mirabilis is a human opportunistic pathogen, which is frequently isolated from intestinal and urinary tract infections, wounds, burns and sepsis [1–3]. The urinary tract infections can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. *Proteus* antigens were reported to be involved in rheumathoid arthritis [4,5]. Cell-surface lipopolysaccharide is considered among potential virulence factors of *Proteus* mediating the infectious processes [6,7]. *Proteus* strains are serologically heterogeneous due to a high diversity of composition and structure of the

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O-specific polysaccharide chain of the lipopolysaccharide (O-antigen). Accordingly, strains of *P. mirabilis* and *P. vulgaris* have been classified into 60 O-serogroups [8,9], and some additional serogroups have been recently proposed for strains of *P. penneri* [10].

Aiming at the understanding of the immunospecificity of *Proteus* on the molecular level and at the creation of the chemical basis for the serological classification, we have elucidated structures of the O-specific polysaccharides of a number of *Proteus* strains [10]. Most of the polysaccharides ($\sim 80\%$) were found to be acidic due to the presence of uronic acids, amino acids, and various other non-carbohydrate acidic components. Now we report the structure of a new acidic O-specific polysaccharide from *P. mirabilis* O11, which is the second *Proteus* O-antigen, after that of *P. penneri* 11 [10,11], found to contain L-threonine.

2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from dried bacterial cells of *P. mirabilis* O11 by the phenol–water procedure [12]. Sugar analysis after acid hydrolysis of the polysaccharide revealed Glc, GlcA and GalA, which were identified using a sugar analyser. Analysis with an amino acid analyser showed the presence of GlcN and threonine in the ratio 2:1. Determination of the absolute configurations of the monosaccharides by GLC of acetylated (+)-2-butyl glycosides showed the D configuration of all of them. The L configuration of threonine was demonstrated by GLC of an acetylated (+)-2-butyl ester.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for five anomeric carbons at δ 99.1–104.3, three carbons bearing nitrogen at δ 55.8–59.0 (C-2 of GlcN and C-2 of Thr), 18 sugar ring carbons bearing oxygen and C-3 of Thr in the region δ 68.8–84.7, two unsubstituted HOCH_2–C groups at δ 61.7 and 62.2 and one substituted group at δ 66.5 (C-6 of Glc and GlcN, data of the attached-proton test [13]), one CH_3–C group at δ 20.0 (C-4 of Thr), three carboxyl groups at δ 171.5, 173.2 and 176.0 (C-6 of GlcA and GalA and C-1 of Thr), and two *N*-acetyl groups (CH_3 at δ 23.5 and 23.9, CO at δ 174.7 and 175.8). In the gated-decoupling ^{13}C NMR spectrum, the signal for C-1 at δ 99.1 displayed a $^1J_{C,H}$ coupling constant of 172 Hz and, hence, belonged to an α-linked sugar. The other C-1 signals were characterised by lower $^1J_{C,H}$ coupling constant values of 161–164 Hz, thus indicating that the remaining monosaccharides are β-linked [14].

The ^{1}H NMR spectrum of the polysaccharide contained signals for five anomeric protons at δ 4.61–4.99, other sugar protons and H-2,3 of Thr at δ 3.42–4.54, H-4 of Thr at δ 1.21 (3 H, d, $J_{3,4}$ 7 Hz), and two *N*-acetyl groups at δ 2.04 and 2.06 (each 3 H, s).

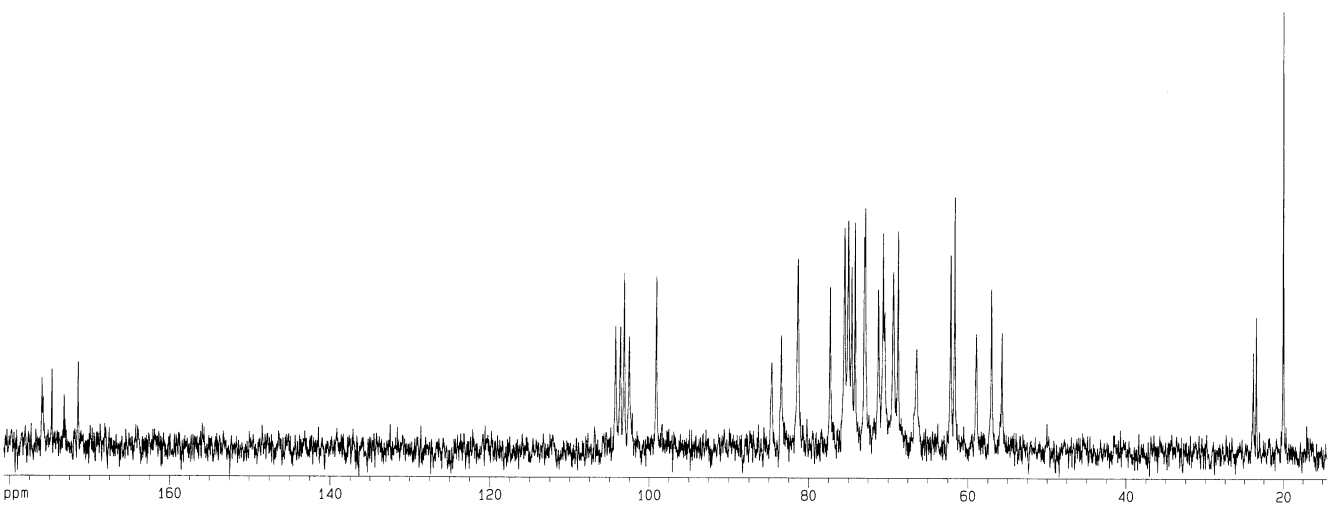


Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide of *P. mirabilis* O11.

Table 1

^{1}H NMR chemical shifts (ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
O-specific polysaccharide[a]							
→3)-β-D-Glc*p*NAc^{I}-(1→	4.61	3.90	3.88	3.74	3.74	4.04	3.80
6 ↑							
→4)-β-D-Glc*p*A-(1→	4.84	3.70	3.71	3.86	3.98		
2 ↑							
→3)-β-D-Gal*p*A6LThr-(1→	4.62	3.70	3.86	4.44	4.30		
β-D-Glc*p*NAc^{II}-(1→	4.82	3.71	3.61	3.42	3.50	3.75	3.95
α-D-Glc*p*-(1→	4.99	3.57	3.81	3.45	3.72	3.77	3.86
Smith-degraded polysaccharide[b]							
→3)-β-D-Glc*p*NAc^{I}-(1→	4.62	3.90	3.88	3.61	3.55	3.80	3.96
→4)-β-D-Glc*p*A-(1→	4.78	3.49	3.69	3.84	3.98		
→3)-β-D-Gal*p*A6LThr-(1→	4.61	3.72	3.91	4.47	4.32		

[a] Additional chemical shifts: NAc at δ 2.04 and 2.06; Thr at 1.21 (Me), 4.40 (C*H*OH) and 4.54 (C*H*NH).

[b] Additional chemical shifts: NAc at δ 2.04, Thr at 1.23 (Me), 4.42 (C*H*OH) and 4.52 (C*H*NH).

Therefore, the polysaccharide has a pentasaccharide repeating unit containing two residues of D-GlcNAc and one residue each of D-Glc, D-GlcA, D-GalA and L-Thr. The characteristic signals at δ 171.5 and 176.0 could be assigned to C-6 of hexuronamide and the free carboxyl group of Thr, respectively (compare published data [11]). Hence, Thr amidates one of the hexuronic acids present in the polysaccharide.

The ^{1}H NMR spectrum of the polysaccharide (Table 1) was assigned using 2D COSY, TOCSY, NOESY, and ^{1}H-detected ^{1}H,^{13}C HMQC experiments. The last experiment also allowed the assignment of the ^{13}C NMR spectrum (Table 2). Based on the $^3J_{H,H}$ coupling constant values, the five sugar spin systems of α-Glc*p*, β-Glc*p*NAc^{I}, β-Glc*p*NAc^{II}, β-Glc*p*A, and β-Gal*p*A were identified. The GlcNAc residues were distinguished from the other sugars with the *gluco* configuration using the ^{1}H,^{13}C HMQC spectrum, which showed correlations of protons at carbons bearing nitrogen (H-2 at δ 3.71 and 3.90) to the corresponding carbons (C-2 at δ 57.1 and 55.8, respectively).

In the ^{13}C NMR spectrum of the polysaccharide, significant downfield displacements were observed for the signals for C-3 of β-GalA, C-2 and C-4 of β-GlcA, C-3 and C-6 of β-GlcNAc^{I} to δ 84.7, 81.4(2), 83.5 and 66.5, respectively, compared with their positions in the spectra of the corresponding unsubstituted monosaccharides [15,16]. These data demonstrated the substitution pattern of the sugar residues in the polysaccharide. The occurrence of two branching points in the repeating unit of the polysaccharide (2,4-disubstituted GlcA

Table 2
^{13}C NMR chemical shifts (ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
O-specific polysaccharide[a]						
→3)-β-D-Glc*p*NAc^{I}-(1→ 6 ↑	102.5	55.8	83.5	69.4	75.1	66.5
→4)-β-D-Glc*p*A-(1→ 2 ↑	103.7	81.4	75.5	81.4	75.1	173.2
→3)-β-D-Gal*p*A6LThr-(1→	104.3	70.5	84.7	69.4	75.5	171.5
β-D-Glc*p*NAc^{II}-(1→	103.2	57.1	74.6	71.3	77.3	62.2
α-D-Glc*p*-(1→	99.1	72.9	74.2	70.7	73.0	61.7
Smith-degraded polysaccharide[b]						
→3)-β-D-Glc*p*NAc^{I}-(1→	102.2	56.0	83.1	69.9	76.8	62.0
→4)-β-D-Glc*p*A-(1→	105.1	74.0	75.0	81.3	75.6	174.3
→3)-β-D-Gal*p*A6LThr-(1→	104.5	70.7	83.3	69.9	75.6	171.5

[a] Additional chemical shifts: NAc at δ 23.5 and 23.9 (Me), 174.7 and 175.8 (CO); Thr at δ 20.0 (Me), 59.0 (CHNH), 68.8 (CHOH) and 176.0 (COOH).

[b] Additional chemical shifts: NAc at δ 23.8 (Me) and 175.1 (CO); Thr at δ 20.2 (Me), 59.3 (CHNH). 68.8 (CHOH) and 176.1 (COOH).

and 3,6-disubstituted GlcNAc^{I}) was confirmed by the ^{13}C chemical shifts for the lateral monosaccharides, α-Glc and β-GlcNAc^{II}, which were close to those in the corresponding non-substituted monosaccharides [15,16].

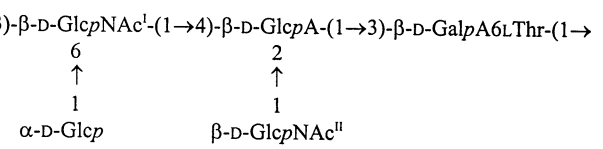
A NOESY experiment showed the following inter-residue cross-peaks: α-Glc H-1, β-GlcNAc^{I} H-5,6a,6b at δ 4.99/3.74, 3.80, 4.04 and β-GlcNAc^{II} H-1, β-GlcA H-2 at δ 4.82/3.70. These data revealed the sites of the attachments of both lateral monosaccharide residues to the main chain of the polysaccharide. The expected connectivities between transglycosidic protons of the sugar residues in the backbone were also observed in the NOESY spectrum, but their assignment was complicated by close resonance positions of H-3 of GlcNAc^{I} and GalA and H-4 of GlcA at δ 3.86–3.88.

To determine the sequence of the monosaccharides in the main chain, the polysaccharide was subjected to a Smith degradation [17]. As a result, a modified polysaccharide was obtained, which was studied by analysis and NMR spectroscopy as described above for the initial polysaccharide. As expected, the modified polysaccharide contained GlcNAc, GlcA, GalA and Thr. The assignments of its ^{1}H and ^{13}C NMR spectra, which were performed using 2D COSY, TOCSY, and ^{1}H,^{13}C HMQC experiments, are given in Tables 1 and 2. The linear character of the Smith-degraded polysaccharide followed from low-field positions of the signals for C-3 of GlcNAc^{I} and

GalA and C-4 of GlcA at δ 83.1, 83.3 and 81.3, respectively. The NOESY spectrum of the modified polysaccharide clearly showed all three expected inter-residue correlations, namely, H-1 β-GlcNAc^{I}, H-4 β-GlcA, H-1 β-GlcA/H-3 β-GalA and H-1 β-GalA/H-3 β-GlcNAc^{I} at δ 4.62/3.84, 4.78/3.91 and 4.61/3.88, respectively. These data defined the sequence and the modes of substitution of the monosaccharides in the main chain of the polysaccharide.

To determine the location of Thr, the ^{1}H NMR spectrum of the Smith-degraded polysaccharide was studied at different pD values. The signal for H-5 of GlcA at δ 3.98 shifted downfield by $\sim$0.1 ppm with the change of pD from 6 to 1, whereas the position of the signal for H-5 of GalA at δ 4.32 was the same at both pD. The downfield displacement is characteristic for H-5 of a hexuronic acid with the free carboxyl group [18], and, hence, Thr is attached to GalA that N-acylates the amino group of the amino acid.

On the basis of the data obtained, it is concluded that the O-specific polysaccharide of *P. mirabilis* O11 has the following structure:



Previously, *N*-(D-galacturonoyl)-L-threonine has been found in the O-specific polysaccharide of *P. penneri* 11 and demonstrated to play a role in the immunodominant group [10,11]. In order to reveal a role of the same component in *P. mirabilis* O11 antigen, rabbit anti-*P. mirabilis* O11 serum was tested in ELISA with homologous and heterologous antigens. It was found that Smith degradation dramatically decreased the reactivity of the homologous lipopolysaccharide (titres before and after Smith degradation were 1:128,000 and 1:8,000, respectively). Therefore, the lateral Glc and GlcNAc residues, which were destroyed by periodate oxidation, are most important for manifesting the *P. mirabilis* O11 immunospecificity, whereas D-galacturonoyl-L-threonine is of minor importance. In accordance with this finding, no significant cross-reactivity was observed in ELISA between anti-*P. mirabilis* O11 serum and *P. penneri* 11 lipopolysaccharide (titre in ELISA 1:1,000). A different contribution of D-galacturonoyl-L-threonine to the specificity of the O-antigens of *P. penneri* 11 and *P. mirablis* O11 may be accounted for by its lateral position in the former, but also by the location in the main chain of the latter polysaccharide, which is decorated by two other lateral sugar residues.

3. Experimental

Isolation of the lipopolysaccharide and O-specific polysaccharide.—The lipopolysaccharide was isolated from dried bacterial cells of *P. mirabilis* O11, strain PrK 24/57, grown as described [19], by extraction with a hot phenol–water mixture [12] and purified by enzymatic treatment [20]. Degradation of the lipopolysaccharide with 0.1 M sodium acetate buffer (pH 4.5) at 100 °C for 1.5 h, followed by GPC on a column (3 × 65 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 5.4) gave the corresponding O-specific polysaccharide.

Sugar analysis.—The polysaccharide was hydrolysed with 3 M CF_3CO_2H (100 °C, 4 h). Amino sugars and amino acids were identified with a Biotronik LC-2000 amino acid analyser at 80 °C using a column (0.4 × 25 cm) of an Ostion LG AN B cation-exchange resin and the standard 0.35 M sodium citrate buffer (pH 5.28). Neutral sugars and hexuronic acids were analysed with a Biotronik LC-2000 sugar analyser at 70 °C using a column (0.4 × 15 cm) of a Dionex A × 8-11 anion-exchange resin and 0.4 M sodium borate buffer (pH 8.0) or 0.02 M potassium phosphate buffer (pH 2.4), respectively. The absolute configuration of the monosaccharide was determined by the published method [21], modified as described [22], using GLC of acetylated (+)-2-butyl glycosides on a Hewlett–Packard 5890 chromatograph equipped with an Ultra 2 capillary column.

Smith degradation.—The O-specific polysaccharide (20 mg) was oxidised in the dark with 1% $NaIO_4$ (20 °C, 40 h), and then 50 mg $NaBH_4$ was added. After 4 h, the solution was acidified with conc AcOH and evapor-

ated. Boric acid was evaporated twice with MeOH, and the material was desalted by GPC on a column (3.5 × 95 cm) of Sephadex G-25 in water. Following hydrolysis with dilute aq HCO_2H (pH 2.1) at 100 °C for 1 h, products were fractionated by GPC on the same gel to give a modified polysaccharide (8.3 mg).

NMR spectroscopy.—^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 35 and 55 °C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process NMR data. A mixing time of 200 ms was used in TOCSY and NOESY experiments.

Preparation of serum and serological assay.—Rabbit O-antiserum against *P. mirabilis* O11 was obtained and ELISA was performed essentially as described earlier [23]. Briefly, a New Zealand rabbit received i.v. increasing doses of the heat-killed bacterium on days 0, 4, 7 and 11; on day 15, 20 mL blood was taken from the ear. In ELISA, rabbit O-antiserum was dispensed to plates containing lipopolysaccharide, and fixed antibodies were quantified with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma).

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