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Note

Structure and cross-reactivity of the O-antigen of *Providencia stuartii* O18 containing 3-acetamido-3,6-dideoxy-D-glucose

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Abstract—The O-polysaccharide (O-antigen) of *Providencia stuartii* O18 was obtained by mild acid degradation of the lipopolysaccharide and studied by chemical methods and NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, NOESY and $^{1}H,^{13}C$ HSQC experiments. The following structure of the tetrasaccharide repeating unit of the polysaccharide was established:

→4)-β-D-Qui*p*3NAc-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*NAc-(1→

where Qui3NAc is 3-acetamido-3,6-dideoxyglucose. Anti-*P. stuartii* O18 serum cross-reacted with the O-antigen of *Proteus* genomospecies 4, which could be accounted for the marked structural similarities of the main chain.

Keywords: Lipopolysaccharide; O-polysaccharide; Bacterial polysaccharide structure; *Providencia stuartii*; *Providencia* O-serogroup

1. Introduction

Bacteria of the genus *Providencia* are facultative pathogens, which are able to invade intestinal mucosa and other cell types and may cause intestinal infections. Particularly, *Providencia stuartii* is a well recognized pathogen that causes urinary tract infections in patients with chronic indwelling urinary catheters.[1] The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii*, which is based on the O-antigens, includes 62 O-serogroups.[2,3] Recently, the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide (LPS) has been established in *Providencia* serogroups O5, O7, O14, O16, O21 and O23 (Ref. 4 and references cited therein). Now we report on the structure and serological specificity of the O-antigen of *P. stuartii* O18.

The polysaccharide was obtained by mild acid degradation of the LPS, isolated from bacterial cells by the phenol–water procedure,[5] followed by GPC on Sephadex G-50. Sugar analysis using GLC of the acetylated alditols revealed GlcN, GalN and 3-amino-3,6-dideoxyglucose (Qui3N) in the ratio ~0.75:0.25:1. Inaddition, glucuronic acid (GlcA) was identified by anion-exchange chromatography using a sugar analyzer. The D configuration of the amino sugars was determined by GLC of the acetylated glycosides with (+)-2-octanol[6,7] and that of GlcA by analysis of the ^{13}C NMR chemical shift data of the polysaccharide (see below). Methylation analysis of the polysaccharide revealed 6-substituted GlcN, 3-substituted GalN and 4-substituted Qui3N residues.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) showed signals for four anomeric carbons at δ

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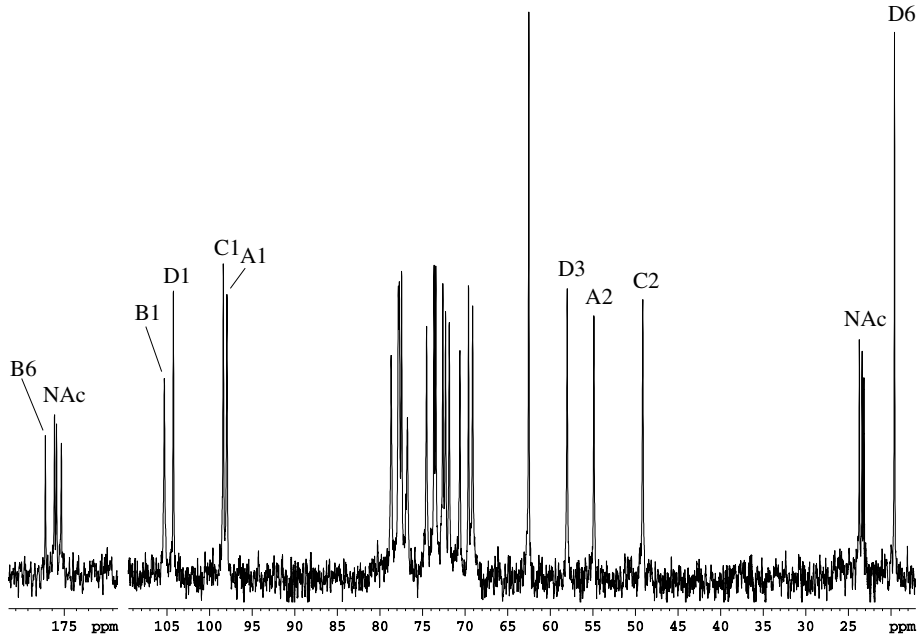

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. stuartii* O18. Arabic numerals refer to carbons of α-Glc*p*NAc (A), β-Glc*p*A (B), α-Gal*p*NAc (C) and β-Qui*p*3NAc (D).

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

	C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-D-Qui*p*3NAc-(1→	104.2	73.6	58.0	77.4	73.4	19.6
→6)-α-D-Glc*p*NAc-(1→	97.9	54.9	71.8	70.6	72.3	69.1
→4)-β-D-Glc*p*A-(1→	105.3	74.5	77.8	77.7	76.7	176.6
→3)-α-D-Gal*p*NAc-(1→	98.4	49.1	78.7	69.6	72.6	62.5
	H-1	H-2	H-3	H-4	H-5	H-6a,6b
→4)-β-D-Qui*p*3NAc-(1→	4.55	3.27	4.05	3.50	3.67	1.37
→6)-α-D-Glc*p*NAc-(1→	5.40	3.87	3.70	3.57	3.80	4.08, 3.90
→4)-β-D-Glc*p*A-(1→	4.48	3.27	3.64	3.73	3.73	
→3)-α-D-Gal*p*NAc-(1→	5.23	4.33	3.85	4.21	4.06	3.73, 3.73

[a]Signals for NAc are at δ_C 23.2, 23.4 and 23.7 (Me), 175.2, 175.6 and 175.8 (CO); δ_H 2.00, 2.02 and 2.04.

97.9–105.3, three nitrogen-bearing carbons (C-2 of GlcN and GalN and C-3 of Qui3N) at δ 49.1–58.0, one methyl group of a 6-deoxy sugar (C-6 of Qui3N) at δ 19.6, one carboxyl group (C-6 of GlcA) at δ 176.6, other sugar carbons in the region δ 62.5–78.7 and three *N*-acetyl groups at δ 23.2–23.7 (Me) and 175.2–175.8 (CO). The absence from the spectrum of signals at δ 82–88 ppm that are characteristic of furanosides[8] indicated that all monosaccharides are in the pyranose form.

The ^{1}H NMR spectrum of the polysaccharide (Table 1) showed, inter alia, signals for four anomeric protons at δ 4.48–5.40, one methyl group of a 6-deoxy sugar (H-6 of Qui3N) at δ 1.37 and three *N*-acetyl groups at δ 2.00–2.04. Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-GlcNAc, D-GalNAc, D-Qui3NAc and D-GlcA. A smaller amount of GalN than expected, which was released by acid hydrolysis, can be accounted for by retention as a GlcA→GalN disaccharide fragment (see below).

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, NOESY and H-detected ^{1}H,^{13}C HSQC experiments (Table 1). The COSY spectrum showed correlations between the neighbouring protons within each spin system. The TOCSY spectrum showed correlations for H-1 with H-2,3,4,5 of GlcNAc, Qui3NAc and GlcA and with H-2,3,4 of GalNAc. The spin systems of amino sugars were distinguished by correlations of protons at nitrogen-bearing carbons with the corresponding carbons at δ 49.1, 54.9 and 58.0.

Intense intraresidue H-1,H-2 cross-peaks in the NOESY spectrum (Fig. 2) showed that GlcNAc and GalNAc are α-linked, whereas H-1,H-3 and H-1,H-5

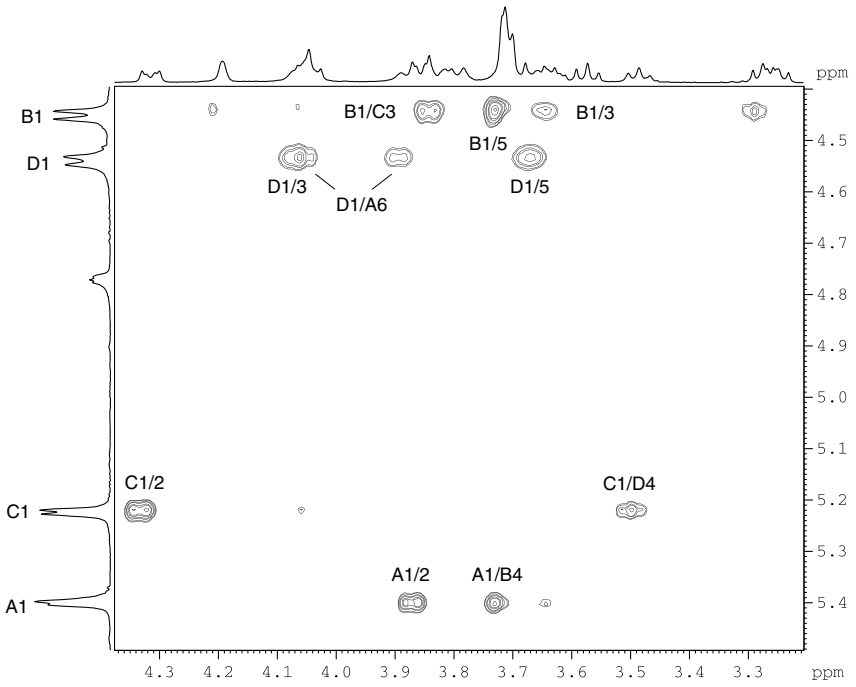

Figure 2. Part of a NOESY spectrum of the O-polysaccharide of *P. stuartii* O18. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons of α-Glc*p*NAc (A), β-Glc*p*A (B), α-Gal*p*NAc (C) and β-Qui*p*3NAc (D).

cross-peaks indicated that Qui3NAc and GlcA are β-linked. The NOESY spectrum showed also strong interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: Qui3NAc H-1,GlcNAc H-6a,6b at δ 4.55/4.08 and 4.55/3.90; GlcNAc H-1,GlcA H-4 at δ 5.40/3.73; GlcA H-1,GalNAc H-3 at δ 4.48/3.85; and GalNAc H1,Qui3NAc H-4 at δ 5.23/3.50. These data defined the glycosylation pattern and the monosaccharides sequence in the repeating unit. The positions of glycosylation sites were confirmed by significant downfield displacements of the signals for GlcNAc C-6, GlcA C-4, GalNAc C-3 and Qui3NAc C-4 to δ 69.1, 77.7, 78.7 and 77.4, respectively, as compared with their positions in the corresponding nonsubstituted monosaccharides.[8–10]

The D configuration of GlcA was demonstrated by a relatively large glycosylation effect (~8 ppm) on C-1 of this sugar, which is characteristic for the same absolute configurations of the monosaccharides in the α-Glc*p*A-(1→4)-D-Gal*p*NAc disaccharide fragment (an effect of ~3 ppm would be observed in case of different absolute configurations of the monosaccharides).[11]

Therefore, it was concluded that the repeating unit of the O-polysaccharide of *P. stuartii* O18 has the structure shown in Figure 3A, which is unique among the known structures of the bacterial O-antigens. Rabbit polyclonal antiserum against heat-killed bacteria of *P. stuartii* O18 reacted strongly with the homologous LPS but showed no or very weak reactivity with the LPS of *Providencia* strains from other serogroups (Table 2). These chemical and serological data are in agreement with classification of *P. stuartii* O18 into a separate *Providencia* O-serogroup.

Providencia stuartii O18

→4)-β-D-Qui*p*3NAc-(1→6)-α-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*NAc-(1→

Proteus genomospecies 4

α-D-Glc*p*
1
↓
2
→4)-β-D-Gal*p*A-(1→3)-α-D-Gal*p*NAc-(1→4)-β-D-Qui*p*3NAc-(1→6)→-β-D-Glc*p*NAc-(1→

Figure 3. Structures of the O-polysaccharides of *Providencia stuartii* O18 (this work) and *Proteus* genomospecies 4 (A. V. Perepelov, K. Zych, Y. Knirel, Z. Sidorczyk, unpublished data).

Table 2. Serological reactivity of anti-*P. stuartii* O18 serum with the LPS of *Providencia* and *Proteus* (reciprocal titre)[a]

Antigen from	Passive haemolysis	EIA
Providencia stuartii O18	25,600	2,048,000
Providencia stuartii O33	200	1000
Providencia alcalifaciens O5	800	2000
Proteus genomospecies 4	3200	32,000
Proteus mirabilis O11	1600	2000

[a] Alkali-treated LPS or LPS was used as antigen in passive haemolysis and EIA, respectively.

Anti-*P. stuartii* O18 serum was tested also with the LPS from various *Proteus* strains in passive haemolysis, enzyme-immunosorbent assay (EIA) and Western blot. A marked cross-reactivity was observed with the LPS of *Proteus* genomospecies 4 (Table 2). Absorption with the LPS of *Proteus* genomospecies 4 reduced the reactivity of anti-*P. stuartii* O18 serum with the homologous LPS and alkali-treated LPS from the titres 2,048,000 and 25,600 to the titres 51,200 and 6400 in EIA and passive haemolysis, respectively. In Western blot (Fig. 4), anti-*P. stuartii* O18 serum recognized both slow and fast migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species with and without O-polysaccharide chain, respectively. It reacted also with high-molecular-mass LPS species of *Proteus* genomospecies 4, thus indicating that a cross-reactive epitope(s) resides on the O-polysaccharide part. Comparison of the O-polysaccharide structures of *P. stuartii* O18 and *Proteus* genomospecies 4 showed a marked similarity in composition, sequence and modes of linkage between the monosaccharides in the main chain (Fig. 3), which is evidently responsible for the observed cross-reactivity.

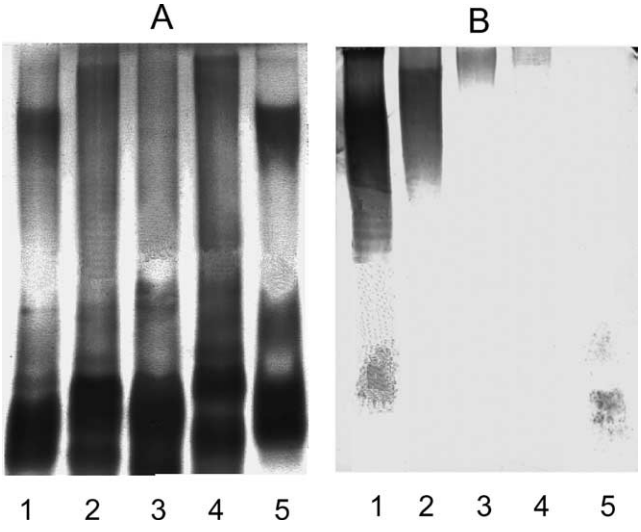

Figure 4. Sodium deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) (A) and Western blot with anti-*P. stuartii* O18 (B) of the LPS of *Providencia stuartii* O18 (lane 1), *Proteus* genomospecies 4 (lane 2), *Providencia stuartii* O33 (lane 3), *P. alcalifaciens* O5 (lane 4) and *Proteus mirabilis* O11 (lane 5).

Western blot revealed also a weak cross-reactivity of anti-*P. stuartii* O18 serum with low-molecular-mass LPS species of *Proteus mirabilis* O11 (Fig. 4). This finding indicated sharing of an epitope(s) on the LPS core of *P. stuartii* O18 and *P. mirabilis* O11, whose structures remain to be determined.

2. Experimental

2.1. Bacterial strain and growth

Providencia stuartii O18:H14 strain H89 came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). The bacteria were cultivated under aerobic conditions in nutrient broth supplemented with 1% glucose. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilized.

2.2. Isolation and degradations of the lipopolysaccharide and the polysaccharide

The lipopolysaccharide was isolated from bacterial cells by phenol–water extraction[4] and purified by treatment with cold aq 50% CCl_3CO_2H; the aqueous layer was dialyzed and freeze-dried. A high-molecular-mass polysaccharide was prepared by degradation of the lipopolysaccharide with aq 2% HOAc at 100 °C for 7 h followed by GPC of the water-soluble portion on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 (4 mL pyridine and 10 mL HOAc in 1 L water) with monitoring the elution using a Knauer differential refractometer. The yield of the polysaccharide was 23.2% of the lipopolysaccharide weight.

2.3. Monosaccharide analysis

The polysaccharide was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h). Amino sugars were converted into the alditol acetates[12] and analyzed by GLC on a Hewlett-Packard 5880 instrument with a DB-5 capillary column using a temperature gradient of 160 °C (3 min) to 290 °C at 10 °C min^{-1}. Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer as described.[4] The absolute configurations of the amino sugars were determined by GLC of the acetylated glycosides with (+)-2-octanol[5,6] under the same chromatographic conditions as above.

2.4. Methylation analysis

Methylation was performed as described.[13] After hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), the partially methylated monosaccharides were reduced with

$NaBH_4$, acetylated and analyzed by GLC–MS on a Hewlett-Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and combined with a NERMAG R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

2.5. NMR spectroscopy

Spectra were recorded using a Bruker DRX-500 spectrometer at pD 2 and 30 °C (1D ^1H NMR and 1D ^{13}C NMR) or 27 °C (2D NMR experiments). Prior to the measurements, the samples were lyophilized twice from D_2O. A mixing time of 200 ms was used in 2D TOCSY and NOESY experiments. Bruker software XWINNMR 2.6 was used to acquire and process the data. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45).

2.6. Serological techniques

Rabbit polyclonal anti-*P. stuartii* O18 serum was obtained by immunization of New Zealand white rabbits with heat-killed bacteria as described.[14] Passive haemolysis with alkali-treated LPS and enzyme-immunosorbent assay (EIA) with LPS as antigen, DOC-PAGE and Western blot were performed as described previously.[15]

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