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Note

Structure of the O-polysaccharide of *Providencia alcalifaciens* O19

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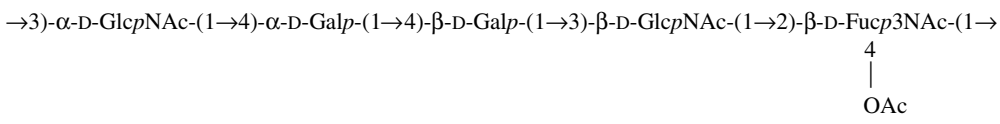
Abstract—Studies of the O-polysaccharide chain of the lipopolysaccharide (O-antigen) of *Providencia alcalifaciens* O19 by sugar and methylation analyses along with NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, NOESY and $^{1}H,^{13}C$ HSQC experiments, showed that the pentasaccharide repeating unit of the polysaccharide has the following structure:



where Fuc3NAc is 3-acetamido-3,6-dideoxygalactose. The unique structure of the O-antigen and serological data are in consistence with classification of this bacterium in a separate *Providencia* serogroup.

Keywords: Lipopolysaccharide; O-polysaccharide; Bacterial polysaccharide structure; *Providencia alcalifaciens*

1. Introduction

Providencia is a genus of *Enterobacteriaceae*, which, together with genera *Proteus* and *Morganella*, is included in the tribe *Proteae*. Strains of three *Providencia* species, *Providencia alcalifaciens*, *P. rustigianii* and *P. stuartii*, are classified in 62 O-serogroups and 30 H-serogroups.[1,2] The serological O-specificity of *Providencia* is defined by the O-polysaccharide chain of the lipopolysaccharide (O-antigen). Recently, the structures of the O-antigens of a number of *Providencia* O-serogroups have been established (Refs. 3, 4 and references cited in Ref. 3). Now we report on the structure and serological properties of the O-antigen of *P. alcalifaciens* O19.

The lipopolysaccharide was isolated from bacterial cells by the phenol–water procedure[5] and degraded by mild acid hydrolysis to give the O-polysaccharide, which was isolated by GPC on Sephadex G-50. Monosaccharide analysis of the polysaccharide revealed Gal, GlcN and 3-amino-3,6-dideoxygalactose (Fuc3N) in the ratios 2:1:0.8 (detector response). The D configuration of the amino sugars was determined by GLC of the acetylated glycosides with (+)-2-octanol. Methylation analysis revealed 4-substituted Gal, 3-substituted GlcN and 2-substituted Fuc3N.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) showed signals for five sugar residues, including

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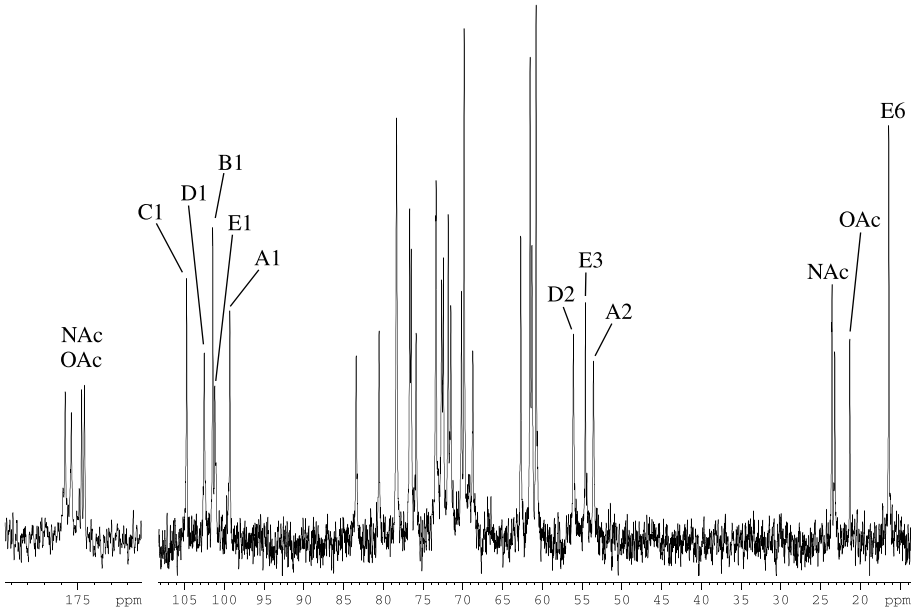


Figure 1. ^{13}C NMR spectrum of the O-specific polysaccharide of *P. alcalifaciens* O19. Arabic numerals refer to carbons of α-Glc*p*NAc (A), α-Gal*p* (B), β-Gal*p* (C), β-Glc*p*NAc (D) and β-Fuc*p*3NAc (E).

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

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-D-Glc*p*NAc-(1→	(A)	99.3	53.5	80.5	68.8	72.5	61.3
→4)-α-D-Gal*p*-(1→	(B)	101.4	69.9	71.5	78.4	72.7	60.8
→4)-β-D-Gal*p*-(1→	(C)	104.7	71.9	73.4	78.4	76.7	61.5
→3)-β-D-Glc*p*NAc-(1→	(D)	102.5	56.1	83.4	70.2	76.5	62.7
→2)-β-D-Fuc*p*3NAc-(1→	(E)	101.2	75.8	54.6	73.4	69.9	16.4
		H-1	H-2	H-3	H-4	H-5	H-6a,6b
→3)-α-D-Glc*p*NAc-(1→	(A)	4.94	4.06	3.98	3.68	4.25	3.83, 3.78
→4)-α-D-Gal*p*-(1→	(B)	4.98	3.90	3.99	4.07	4.39	3.66, 3.61
→4)-β-D-Gal*p*-(1→	(C)	4.50	3.55	3.70	4.04	3.75	3.89, 3.83
→3)-β-D-Glc*p*NAc-(1→	(D)	4.59	3.72	3.84	3.44	3.42	3.94, 3.71
→2)-β-D-Fuc*p*3NAc-(1→	(E)	4.76	3.70	4.21	5.05	3.99	1.14

Signals for NAc are at δ_C 23.2, 23.6, 23.6 (Me), 174.6, 175.4 and 175.7 (CO); δ_H 1.95 and 2.01; for OAc at δ_C 21.3 (Me) and 174.8 (CO); δ_H 2.13.

signals for five anomeric carbons at δ 99.3–104.7, three nitrogen-bearing carbons (C-2 of two GlcN residues and C-3 of Fuc3N) at δ 53.5–56.1, one methyl group of a 6-deoxy sugar (C-6 of Fuc3N) at δ 16.4, other sugar carbons in the region δ 60.8–83.4, three *N*-acetyl groups (Me at δ 23.2–23.6), one *O*-acetyl group (Me at δ 21.3) and four CO groups at δ 174.6–175.7. Accordingly, the ^{1}H NMR spectrum of the polysaccharide (Table 1) showed, inter alia, signals for five anomeric protons at δ 4.50–4.98, one methyl group of a 6-deoxy sugar (H-6 of Fuc3N) at δ 1.14, three *N*-acetyl groups at δ 1.95 and 2.01, and one *O*-acetyl group at δ 2.13. Therefore, the polysaccharide has a pentasaccharide repeating unit containing two residues each of D-GlcNAc and D-Gal and one residue of D-Fuc3NAc as well as an *O*-acetyl group.

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 and spin systems for five monosaccharide residues were identified (Table 1). GlcNAc residues were distinguished by a relatively large $J_{3,4}$ and $J_{4,5}$ coupling constant values of ~9 Hz as compared with those of <3 Hz in Gal and Fuc3NAc residues. Fuc3NAc was identified by correlation of the proton at the nitrogen-bearing carbon (H-3) with the corresponding carbon (C-3) at δ 54.6. The presence in the NOESY spectrum (Fig. 2) of intense intraresidue H-1,H-2 cross-peaks showed that one of the residues of GlcNAc and Gal are α-linked, whereas H-1,H-3 and H-1,H-5 cross-peaks indicated that the three other monosaccharide residues are β-linked.

The NOESY spectrum of the polysaccharide (Fig. 2) showed also interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: α-GlcNAc H-1,α-Gal H-4 at δ 4.94/4.07, α-Gal H-1,β-Gal H-4 at δ 4.98/4.04, β-Gal H-1,β-GlcNAc H-3

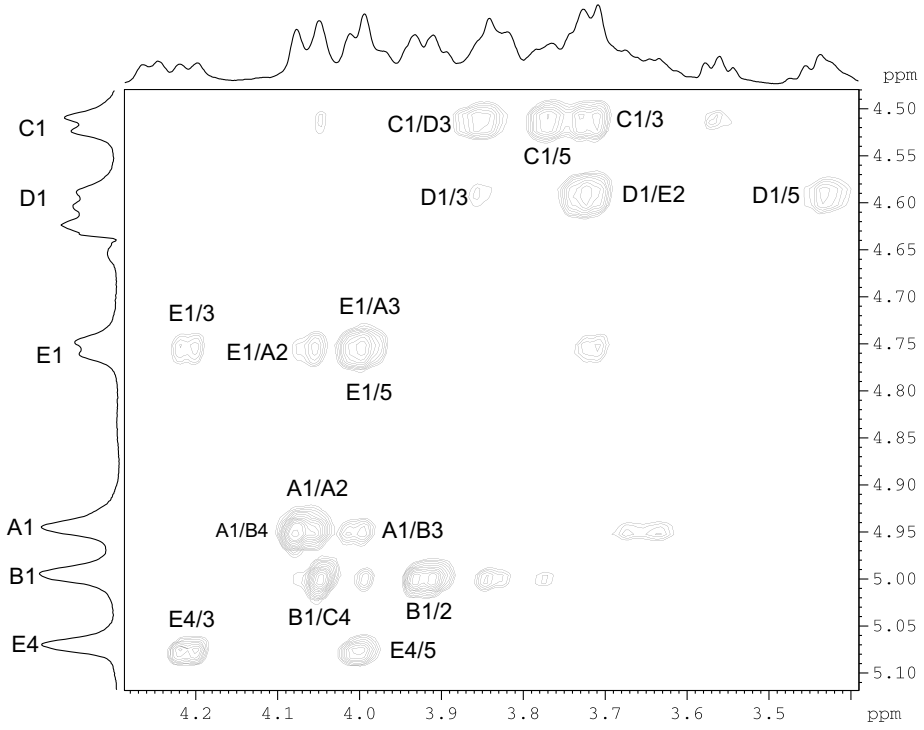


Figure 2. Part of a NOESY spectrum of the O-specific polysaccharide of *P. alcalifaciens* O19. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons of α-Glc*p*NAc (A), α-Gal*p* (B), β-Gal*p* (C), β-Glc*p*NAc (D) and β-Fuc*p*3NAc (E).

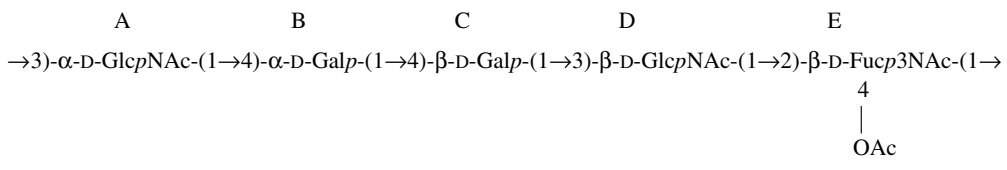
at δ 4.50/3.84, β-GlcNAc H-1,Fuc3NAc H-2 at δ 4.59/3.70 and Fuc3NAc H-1,α-GlcNAc H-3 at δ 4.76/3.98. These data defined the glycosylation pattern and the monosaccharide sequence in the repeating unit. The modes of substitution of the monosaccharides were confirmed by down-field displacement of the signals for linkage carbons to δ 75.8–83.4 (Table 1), as compared with their positions in the corresponding nonsubstituted monosaccharides.[6] The position of *O*-acetylation followed from a down-field displacement of the H-4 of Fuc3NAc signal to δ 5.05 due to deshielding effect of the *O*-acetyl group.

Therefore, it was concluded that the repeating unit of the O-polysaccharide of *P. alcalifaciens alvalifaciens* O19 has the following structure:

Table 2. Serological reactivity of anti-*P. alcalifaciens* O19 serum with LPS of *Providencia* (reciprocal titer)[a]

Antigen from	Passive haemolysis	EIA
P. alcalifaciens O19	25,600	2,048,000
P. alcalifaciens O3	1600	32,000
P. alcalifaciens O8	400	8000
P. alcalifaciens O21	800	8000

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



The lipopolysaccharide from *P. alcalifaciens* O19 and those from various *Providencia* and *Proteus* strains that have structurally similar O-antigens were tested with rabbit polyclonal antiserum against heat-killed bacteria of *P. alcalifaciens* O19. The O-antiserum gave a strong serological reaction with the homologous lipopolysaccharide in passive haemolysis and enzyme immunosorbent assay (Table 2). A weak cross-reactivity was observed with *P. alcalifaciens* O3, O8 and O21 (Table 2). Comparison of the O-polysaccharide structure of *P. alcalifaciens* O19 established in this work with that of *P. alcalifaciens* O21 reported earlier[3] showed that they both contain *N*-acyl derivatives of Fuc3N (*N*-acetyl in the former and *N*-formyl in the latter polysaccharide). Fuc3N is the only sugar in common that may be responsible for the cross-reactivity of strains of these two O-serogroups. Elucidation of the molecular basis of the serological relatedness with *P. alcalifaciens* O3 and

O8 requires determination of the O-polysaccharide structures of these strains, which remain unknown. Taking account of only a weak serological cross-reactivity, it was concluded that the classification of *P. alcalifaciens* O19 in a separate *Providencia* O-serogroup is in agreement with the unique structure and serological properties of the O-antigen of this strain.

2. Experimental

2.1. Bacterial strain and growth

P. alcalifaciens O19:H2 strain 691 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 lyophilised.

2.2. Isolation and degradations of the lipopolysaccharide and the polysaccharide

The lipopolysaccharide was isolated from bacterial cells by phenol–water extraction[5] and purified by treatment with cold aq 50% CCl_3CO_2H; the aqueous layer was dialysed and freeze-dried. A high-molecular-mass polysaccharide was prepared by degradation of the lipopolysaccharide with NaOAc buffer pH 4.2 (100 °C, 9 h) and then with aq 2% HOAc at 100 °C for 1 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 13.0% of the lipopolysaccharide weight.

2.3. Monosaccharide analysis

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Amino sugars were converted into the alditol acetates[7] and analysed 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}. The absolute configurations of the amino sugars were determined by GLC of the acetylated glycosides with (+)-2-octanol[8,9] under the same chromatographic conditions as above.

2.4. Methylation analysis

Methylation was performed as described.[10] After hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analysed 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 37 °C (1D ^{1}H NMR and 2D NMR experiments) or 30 °C (1D ^{13}C NMR). Prior to the measurements, the samples were lyophilised 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. alcalifaciens* O19 serum was obtained by immunisation of New Zealand white rabbits with heat killed bacteria as described.[11] Passive immunohaemolysis with alkali-treated lipopolysaccharides and enzyme immunosorbent assay with lipopolysaccharides as antigen were performed as described previously.[12]

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