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Carbohydrate Research 333 (2001) 179–183

CARBOHYDRATE RESEARCH

Note

Structural studies of the O-polysaccharide from the *Escherichia coli* O77 lipopolysaccharide

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Received 27 November 2000; accepted 24 April 2001

Abstract

The structure of the O-antigen polysaccharide (PS) from *Escherichia coli* O77 has been determined. Sugar and methylation analysis together with ^{1}H and ^{13}C NMR spectroscopy were the main methods used. The PS is composed of tetrasaccharide repeating units with the following structure:

→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→6)-α-D-Man*p*-(1→

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Keywords: *Escherichia coli*; Lipopolysaccharide; NMR

Escherichia coli is a facultative anaerobic Gram negative rod and is a predominant species in the colonic flora of animals and man. The species is subdivided into serotypes based on the immunogenicity of bacterial surface structures. Thus, the strains are usually designated as O:K:H serotypes where O is the O-antigen, i.e., the polysaccharide portion of the lipopolysaccharide; K is the capsular polysaccharide and H the flagella antigen. As of today, more than 170 different O-antigens and over 100 capsular polysaccharides have been identified within the species.[1] There are three general clinical syndromes that result from infections with pathogenic *E. coli*: (i) enteric/diarrhoeal, (ii) urinary tract infections, and (iii) septicaemia/meningitis. Only a limited number of O, K and H antigens and O:K:H serotypes are represented as pathogens in the different infections. The diarrhoeal strains can be further divided into different virotypes based on the type of virulence factors they express and hence on the diarrhoeal disease they cause. These are: enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC).[2] The *E. coli* O77 is an *O*-serotype that may cause different diarrhoeal infections. It has been designated as ETEC since the *E. coli* O77:H7 has been found to produce the heat-stable enterotoxin[3] or as EAEC since *E. coli* O77:H18 has been found positive in the assay for this virotype.[2] In several studies, *E. coli* O77 strains producing

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PII: S0008-6215(01)00123-9

Shiga-like toxin have been isolated both from humans as well as from animals.[4–7] In addition, it has been shown earlier that the *E. coli* O77 cross-reacts with *E. coli* O44.[8] In this study, we describe the structure of the *E. coli* O77 O-polysaccharide revealing the basis for the observed cross-reactivity.

The *E. coli* O77:K96:H$^-$ was grown in a glucose-containing TY medium. The LPS was isolated from the bacterial membrane by hot phenol/water extraction and delipidated under mild acidic conditions to yield a polysaccharide (PS). A hydrolysate of the PS contained mannose and 2-amino-2-deoxyglucose in the ratio 2.7:1. Minor amounts of glucose and heptose were also detected and these components were attributed to the core. Determination of the absolute configuration of the two major components revealed that both had the D-configuration.

Methylation analysis showed the presence of three components, namely, 3,4,6-tri-*O*-methyl-mannose, 2,3,4-tri-*O*-methyl-mannose and 4,6-di-*O*-methyl-*N*-methyl-glucosamine in the ratio 1.4:1:1.2. These derivatives originate from pyranosides of 2-substituted mannose, 6-substituted mannose and 3-substituted glucosamine. The ^{1}H NMR spectrum showed the presence of a methyl signal from an *N*-acetyl group (δ 2.06, 3 H) revealing that the glucosamine residue is *N*-acetylated. In the region for anomeric resonances four signals were present (Fig. 1) indicating that the PS consists of a tetrasaccharide repeating unit. The resonance at δ 4.88 had $J_{\text{H-1,H-2}}$ 3.5 Hz showing that this is the α-linked *N*-acetyl-glucosamine residue. The residues with anomeric resonances at δ 5.32 and 5.05 both had $J_{\text{H-1,C-1}} > 170$ Hz showing that these residues also are α-linked. Finally, the residue with its anomeric resonance at δ 4.76 had $J_{\text{H-1,C-1}}$ 161 Hz showing that this residue is β-linked.

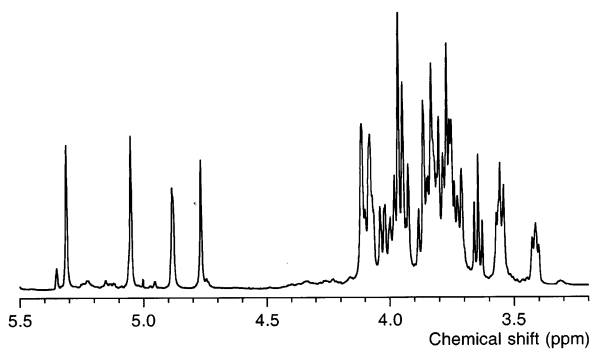

Fig. 1. Part of the ^{1}H NMR spectrum of the O-antigen polysaccharide from *E. coli* O77.

The ^{1}H and ^{13}C NMR spectra were assigned using two-dimensional NMR techniques and the chemical shifts are compiled in Table 1. The ^{13}C NMR glycosylation shifts[9] reveal the substitution pattern of the sugar residues in agreement with the above methylation analysis. Thus, residue **A** is a 2-substituted α-linked mannose, residue **B** is a 6-substituted α-linked mannose, residue **C** is a 3-substituted α-linked *N*-acetyl-glucosamine and residue **D** is a 2-substituted β-linked mannose. The sequence of the sugar residues in the repeating unit was determined from ^{1}H,^{1}H NOESY and ^{1}H,^{13}C HMBC experiments. The connectivities are given in Table 1, from which the sequence –**A**–**D**–**C**–**B**– can be deduced. Therefore, the structure of the O-antigen polysaccharide from *E. coli* O77 is:

→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)
-α-D-Glc*p*NAc-(1→6)-α-D-Man*p*-(1→

It may be noted that besides the *trans*-glycosidic ^{1}H,^{1}H NOE from H-1 in **A** to H-2 in **D** an NOE is also observed from H-1 in **A** to H-5 in **B**. This is in complete agreement with the geometry for the **B**–**A** sequence, i.e., α-D-Man*p*-(1→2)-α-D-Man*p*. The equivalent NOE is also observed in the disaccharide α-L-Rha*p*-(1→2)-α-L-Rha*p*-OMe.[10]

Cross-reactivity has been documented between *E. coli* O77 and *E. coli* O44.[8] We previously determined the O-antigen structure of the latter,[11] which has the pentasaccharide repeating unit shown below:

→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-α-D-Glc*p*NAc-(1→6)-α-D-Man*p*-(1→
4
↑
1
α-D-Glc*p*

Thus, the backbone of the *E. coli* O44 O-antigen is the same as for the *E. coli* O77 O-antigen explaining the observed cross-reactivity.

Table 1
^{1}H and ^{13}C NMR chemical shifts (ppm) of the signals from the O-antigen polysaccharide of *E. coli* O77 and inter-residue correlations from NOESY and HMBC spectra

Sugar residue	$^{1}H/^{13}C$							
	1	2	3	4	5	6	Connectivity to atom (from anomeric atom)	
							NOE	HMBC
→2)-α-D-Man*p*-(1→	5.32	4.08	4.03	3.77	3.99	~3.84	3.97, **D** H-2	76.3, **D** C-2
A	(0.14) [a]	(0.14)	(0.17)	(0.09)	(0.17)		3.82, **B** H-5	
	99.9 [175] [b]	78.8	70.4	67.0	73.0	61.2		3.97, **D** H-2
	(5.0)	(7.1)	(−0.8)	(−0.9)	(−0.3)	(−0.8)		
→6)-α-D-Man*p*-(1→	5.05	4.12	3.86	3.97	3.82	3.55, 4.11	4.08, **A** H-2	78.8, **A** C-2
B	(−0.13)	(0.18)	(0.00)	(0.29)	(0.00)			
	103.0 [172]	70.4	71.2	66.4	72.0	65.4		4.08, **A** H-2
	(8.1)	(−1.3)	(0.0)	(−1.5)	(−1.3)	(3.4)		
→3)-α-D-Glc*p*NAc-(1→ [c]	4.88	4.08	3.95	3.56	3.76	3.80, 3.88	3.55, **B** H-6	65.4, **B** C-6
C	(−0.33)	(0.20)	(0.20)	(0.07)	(−0.10)			
	97.6 [175]	53.5	80.6	68.8	72.3	61.1		
	(5.8)	(−1.5)	(8.9)	(−2.5)	(−0.2)	(−0.7)		
→2)-β-D-Man*p*-(1→	4.76	3.97	3.72	3.65	3.42	3.76, 3.94	3.95, **C** H-3	80.6, **C** C-3
D	(−0.13)	(0.02)	(0.06)	(0.05)	(0.04)			
	100.4 [161]	76.3	74.3	67.3	77.3	61.4		3.95, **C** H-3
	(5.8)	(4.2)	(0.3)	(−0.4)	(0.3)	(−0.6)		

[a] Chemical shift differences as compared to the corresponding monosaccharides.[25]
[b] $J_{H\text{-}1,C\text{-}1}$ values are given in Hz in square brackets.
[c] Chemical shifts for NAc are δ_H 2.06; δ_C 22.6 and 174.2.

1. Experimental

Bacterial strain and growth conditions.—The *E. coli* O77:K96:H$^-$ strain CCUG 11379, was obtained from the Culture Collection University of Gothenburg, Sweden. Bacteria were grown in submerged culture to late exponential phase in 22 L of a tryptone/yeast extract medium[12] containing 1% glucose, using a 30 L fermentor (Belach AB) under constant aeration at 37 °C and pH 7.0. A preculture (3 L) in the same medium was used to inoculate the fermentor. All cultures were checked for purity at the end of the growth cycle. The bacteria were killed with 1% (mass/vol.) formaldehyde. After incubation overnight at 4 °C the cells were separated from the media by continuous-flow centrifugation using a CEPA model LE centrifuge at a cylinder speed of 35,000 rpm and a flow of 25 L/h (Carl Padberg Centrifugenbau). The bacterial mass was then removed from the cylinder, washed once with phosphate-buffered saline (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 20 min, 4 °C) and finally re-suspended in distilled water.

Preparation of lipopolysaccharide and lipid-free polysaccharide.—The LPS was extracted by the hot phenol/water method.[13] The aqueous phase was dialysed at 4 °C for 3–5 days against tap water, then overnight against distilled water, concentrated under diminished pressure and lyophilised. Contaminating nucleic acids were removed by ultracentrifugation (100,000*g*, 4 h, 4 °C). The nucleic acid content was determined spectrophotometrically as described[14] and the protein content was estimated according to Lowry et al. with BSA as standard.[15] The presence of nucleic acid and proteins were found to be $< 5\%$ and $< 0.5\%$, respectively.

Lipid-free polysaccharide (PS) was prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100 °C for 5 h.[16] Lipid A was removed by centrifugation (10,000*g*, 20 min, 4 °C). The PS was further purified by gel-permeation chromatography.

Component analyses.—The PS was hydrolysed with 4 M HCl at 120 °C for 20 min. After reduction with $NaBH_4$ and acetylation, the sample was analysed by GLC. The absolute configuration of the sugars present in the PS were determined by derivation of the sugars as their acetylated (+)-2-butyl glycosides.[17,18]

Methylation analysis.—The analysis was performed according to Hakomori[19] using sodium methylsulfinylmethanide and iodomethane in dimethyl sulfoxide. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore).[20] The purified methylated sample was then hydrolysed (4 M HCl at 120 °C for 20 min), reduced and acetylated. The partially methylated alditol acetates were analysed by GLC–MS.

GLC and GLC–MS analyses.—Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused silica column (0.20 mm × 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C/min to 210 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph equipped with a flame-ionisation detector. GLC–MS analysis was performed on a Thermo Quest GCQ plus spectrometer equipped with a DB-5 fused silica column (0.32 mm × 15 m). A temperature program of 170 °C for 3 min followed by 3 °C/min to 250 °C was used with Helium as carrier gas.

NMR spectroscopy.—NMR spectra of the PS in D_2O were recorded at 45 °C using Varian Inova 400 and 600 MHz instruments. Chemical shifts are reported in ppm relative to sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propanoate (TSP), δ_H 0.00 or dioxan, δ_C 67.4 as internal and external references, respectively. Data processing was performed using standard Varian VNMR software. ^1H,^1H-correlated spectroscopy (COSY),[21] total correlation spectroscopy (TOCSY)[22] with mixing times of 30, 60 and 90 ms, gradient selected heteronuclear single quantum coherence (gHSQC),[23] and gradient selected heteronuclear multiple-bond correlation (gHMBC)[23] experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY)[24] experiment with a mixing time of 50 ms, and an HMBC experiment with a 60 ms delay for the evolution of long-range couplings were used.

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Agency for Research Cooperation with Developing Countries.

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