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Structural determination of the O-antigenic polysaccharide from the Shiga toxin-producing *Escherichia coli* O171

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Abstract—The structure of the O-antigenic part of the lipopolysaccharide (LPS) obtained from the verotoxin-producing *Escherichia coli* O171 has been determined. ^{1}H and ^{13}C NMR spectroscopy techniques in combination with component analysis were used to elucidate the O-antigen structure of O-deacylated LPS. Subsequent NMR analysis of the native LPS revealed acetylation at O-7/O-9 of the sialic acid residue. The sequence of sugars was determined by inter-residue correlations in ^{1}H,^{1}H-NOESY and ^{1}H,^{13}C-heteronuclear multiple-bond correlation spectra. The O-antigen is composed of pentasaccharide repeating units with one equivalent of *O*-acetyl groups distributed over two positions:

→4)-α-Neu5Ac7,9Ac-(2→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→

Based on biosynthetic considerations, this should also be the biological repeating unit.

Keywords: *Escherichia coli*; Verotoxin; Lipopolysaccharide; Sialic acid; NMR

1. Introduction

Among *Escherichia coli* strains causing diarrhoeal diseases, there are six well-described categories: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enterohemorrhagic *E. coli* (EHEC); enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC).[1] These categories have virulence attributes that help bacteria to cause diseases by different mechanisms.

EHEC is an etiological agent of diarrhoea with life-threatening complications like haemorrhagic colitis (HC) and haemolytic–uraemic syndrome (HUS). EHEC belongs to a group of *E. coli* called VTEC ('verotoxigenic *E. coli*') or STEC ('Shiga toxin-producing *E. coli*'). The pathological lesions associated with HC and HUS are due to the action of Shiga toxin (Stx) on endothelial cells. Whereas not all STEC strains are believed to be pathogens, all EHEC strains are considered to be pathogens. The most notorious *E. coli* serotype associated with EHEC is O157:H7, which has been the cause of several large outbreaks of disease in North America, Europe, and Japan.[2–6] The principle reservoir of STEC strains is the bovine intestinal tract, and most outbreaks of EHEC are associated with the consumption of undercooked meat, sausages, unpasteurized milk, lettuce, and apple juice. Besides *E. coli* O157:H7, several other serotypes have been associated with the STEC category. *E. coli* O171 has recently been shown in several studies to be present in cattle and in foods in Argentina and Spain.[7–13] Most of the O171 strains were of the O171:H2 serotype, and all of them possessed the *stx* gene encoding the shiga toxin. In this paper, we present the structure of the O-antigen of the LPS from *E. coli* O171.

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2. Results and discussion

The LPS from *E. coli* O171, grown in a glucose-containing tryptone/yeast extract medium,[14] was isolated from the bacterial membrane by hot phenol/water extraction.[15] Delipidation under mild acidic conditions led to a heterogeneous material not suitable for further structural studies. Our previous investigation of the O-antigen from *E. coli* O164 indicated that it should be possible to determine the O-antigen structure without resorting to acidic treatment of the material.[16] The ^{1}H NMR spectrum of the LPS indicated that *O*-acetyl groups were present in the polymer since resonances at ~2.12 ppm were observed. Subsequent O-deacylation using dilute aqueous ammonia resulted in a material of good quality. The ^{1}H NMR spectrum of the O-deacylated LPS showed the presence of four signals in the anomeric region at δ_H 4.65, 4.57, 4.48, and 4.40 (Fig. 1). Additional resonances were found, inter alia, at δ_H 2.95 and 1.76 as well as at δ_H 2.03 (3H) and 2.00 (3H) indicating a methylene group and two *N*-acetyl groups, respectively, as part of one or more residues in the polymer.

A hydrolysate of the LPS contained glucose, galactose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, and heptose in the ratio 36:43:5:14:5. Sialic acid was shown by NMR spectroscopy (vide infra) to be a component of the polymer. Determination of the absolute configuration of the hexoses in the O-antigen revealed that they had the D-configuration. However, although it is possible to identify sialic acid as its methyl glycoside methyl ester by GLC–MS analysis,[17,18] this was not possible in the present case. The absolute configuration of the sialic acid is assumed to be that previously found in nature, viz., the D-*glycero*-D-*galacto* configuration.[19]

The ^{1}H,^{13}C-HSQC spectrum of the O-deacylated LPS showed in the region for anomeric resonances four peaks corresponding to hexopyranosyl residues

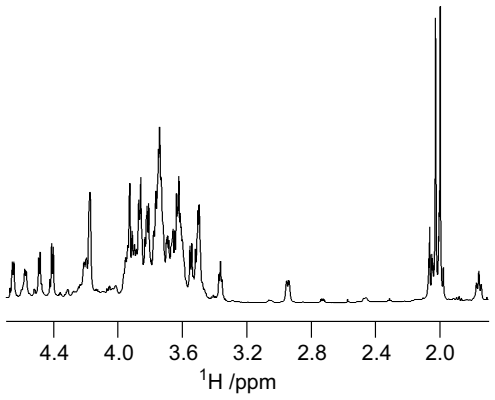

Figure 1. ^{1}H NMR spectrum of the O-deacylated LPS from *E. coli* O171.

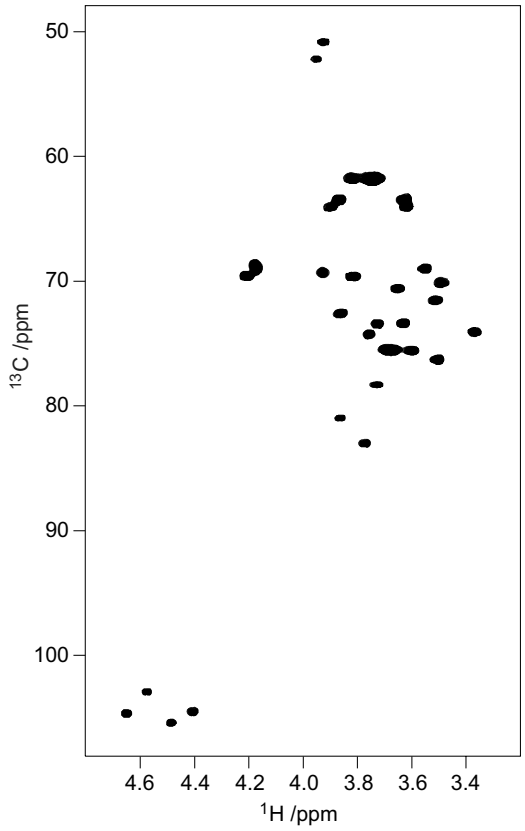

Figure 2. Part of the ^{1}H,^{13}C-HSQC spectrum of the O-deacylated LPS from *E. coli* O171.

(Fig. 2), and since $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}1,C\text{-}1}$ coupling constants of ~8 Hz and ~165 Hz, respectively, were observed for the anomeric protons these residues are β-linked. The ^{1}H and ^{13}C NMR resonances were assigned using 2D NMR techniques and the chemical shifts are compiled in Table 1, in which the sugar residues are referred to as **A**–**D**, in decreasing chemical shift of their anomeric proton. The sialic acid residue, with a chemical shift of 101.2 ppm for its anomeric carbon, was denoted residue **E**. The presence of five anomeric carbons indicated that the O-antigen consists of pentasaccharide repeating units. From the chemical shifts and coupling constants given in Table 1 it can be concluded that all sugar residues have the pyranoid ring form. Residues **B** and **E** carried *N*-acetyl groups and these were assigned from a ^{1}H,^{13}C-HMBC spectrum.

The spin system of **A** having the anomeric resonance at δ_H 4.65 could be assigned to a →6)-β-D-Glc*p*-(1→ residue due to the low chemical shift of H-2 and a large glycosylation shift[20] of C-6, $\Delta\delta_C$ 7.9. Residue **B** with $\delta_{H\text{-}1}$ 4.57 was assigned to a →3)-β-D-Gal*p*NAc-(1→ residue due to the chemical shift of C-2 at 52.2 ppm and a large glycosylation shift of C-3, $\Delta\delta_C$ 9.1. The ^{1}H,^{1}H spin system of **C** with $\delta_{H\text{-}1}$ 4.48 revealed that it had the *galacto*-configuration and from $\Delta\delta_{C\text{-}3}$ 9.2 that it is a →3)-β-D-Gal*p*-(1→ residue. Residue **D** also has

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the signals from the O-antigen part of the O-deacylated LPS from *E. coli* O171

Sugar residue	$^{1}H/^{13}C$										
	1	2	3	4	5	6	7	8	9	Me	CO
→6)-β-D-Glc*p*-(1→ **A**	4.65 [7.8]	3.36	3.50	3.50	3.59	3.81, 4.21					
	(0.01)	(0.11)	(0.00)	(0.08)	(0.13)						
	104.7{165}	74.1	76.3	70.2	75.6	69.7					
	(7.9)	(−1.1)	(−0.5)	(−0.5)	(−1.2)	(7.9)					
→3)-β-D-Gal*p*NAc-(1→ **B**	4.57 [8.3]	3.95	3.86	4.18	3.69	3.74, 3.82				2.00	
	(−0.11)	(0.05)	(0.09)	(0.20)	(−0.03)						
	102.9 {165}	52.2	81.1	68.7	75.5	61.8				23.2	175.2
	(6.6)	(−2.6)	(9.1)	(−0.1)	(−0.5)	(−0.1)					
→3)-β-D-Gal*p*-(1→ **C**	4.48 [7.9]	3.65	3.77	4.17	3.66	~3.74					
	(−0.05)	(0.20)	(0.18)	(0.28)	(0.01)						
	105.4 {165}	70.6	83.0	69.1	75.5	61.8					
	(8.0)	(−2.4)	(9.2)	(−0.6)	(−0.4)	(0.0)					
→6)-β-D-Gal*p*-(1→ **D**	4.40 [7.8]	3.51	3.62	3.93	3.75	3.61, 3.90					
	(−0.13)	(0.06)	(0.03)	(0.04)	(0.10)						
	104.5 {164}	71.6	73.5	69.4	74.3	64.1					
	(7.1)	(−1.4)	(−0.3)	(−0.3)	(−1.6)	(2.3)					
→4)-α-Neu5Ac-(2→ **E**			1.76, 2.95	3.73	3.92	3.72	3.54	3.86	3.62, 3.86	2.03	
			(0.15, 0.22)[a]								
	174.2	101.2	40.8	78.4	50.8	73.5	69.0	72.6	63.5	23.0	175.0
	(0.7)	(0.4)[b]	(0.6)	(10.1)	(−1.3)	(0.8)	(0.7)	(0.8)	(0.7)		

$J_{H\text{-}1,H\text{-}2}$ values are given in Hz in square brackets and $J_{H\text{-}1,C\text{-}1}$ values in braces. Chemical shift differences as compared to the corresponding monosaccharides are given in parentheses.

[a] pD 7.

[b] Methyl α-Neu5Ac.

the *galacto*-configuration as determined from $^{1}H,^{1}H$ correlations but a smaller glycosylation shift of C-6, $\Delta\delta_C$ 2.3, revealing it as a →6)-β-D-Gal*p*-(1→ residue. Finally, residue **E** is the *N*-acetyl-neuraminic acid. It is α-linked since there is a large chemical shift separation of the H-3 protons ($\Delta\delta_H$ 1.19).[19] In addition, a three-bond correlation between C-1 and H-3ax was readily observed in the $^{1}H,^{13}C$-HMBC spectrum, indicating a large value of $J_{C\text{-}1,H\text{-}3ax}$ consistent with an axial carboxylate group, as shown in a previous study.[21] The $^{1}H,^{1}H$-TOCSY based experiments revealed correlations from H-3 to H-6 and from H-7 to H-9 in residue **E**. It has been reported that complete assignments of the proton chemical shifts of Neu5Ac via $^{1}H,^{1}H$-TOCSY experiments is difficult due to the low value of $J_{H\text{-}6,H\text{-}7}$.[22] In our case, the $^{1}H,^{1}H$-NOESY spectrum exposed a cross-peak at 3.54/3.72 ppm corresponding to a correlation between H-7 and H-6, respectively, thus resolving the above described limitations. In addition, the $^{1}H,^{1}H$-NOESY spectrum showed a cross-peak at 3.54/2.03 ppm between H-7 and the methyl protons of the *N*-acetyl group at C-5. That **E** is 4-substituted is evident from a large glycosylation shift of C-4, $\Delta\delta_C$ 10.1. Consequently, the fifth residue in the O-antigen repeating unit is a →4)-α-Neu5Ac-(2→ residue.

The sequence of the sugar residues in the O-antigen repeating unit was determined from $^{1}H,^{1}H$-NOESY (Fig. 3) and $^{1}H,^{13}C$-HMBC experiments (Table 2), from

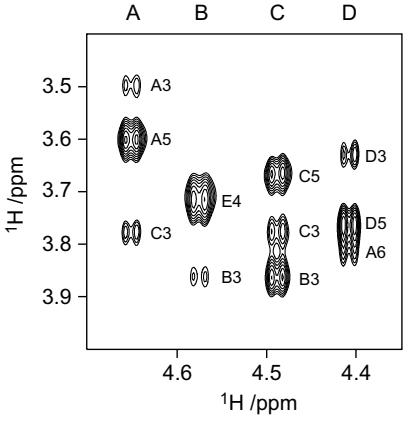

Figure 3. Part of the $^{1}H,^{1}H$-NOESY spectrum of the O-deacylated LPS from *E. coli* O171.

which the sequence **D**–**A**–**C**–**B**–**E** can be deduced. The small glycosylation shift of C-6 in **D**, $\Delta\delta_C$ 2.3, is typical for the substitution with a keto-sugar,[23] thereby confirming the final structural element **E**–**D**. O-Antigen polysaccharides of *E. coli* have been described to contain an *N*-acetylglucosamine or *N*-acetylgalactosamine residue at the reducing end of the biological repeating unit.[24–27] In *E. coli* O171, the biological repeating unit of its O-antigen polysaccharide should have the following structure, which consequently has the sialic acid as the terminal residue:

→4)-α-Neu5Ac-(2→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→

E **D** **A** **C** **B**

Table 2. Inter-residue correlations observed in the $^{1}H,^{1}H$-NOESY and $^{1}H,^{13}C$-HMBC NMR spectra from the O-antigen part of the O-deacylated LPS from *E. coli* O171

Residue	Anomeric atom	Residue	NOE	HMBC
A	H-1	**C**	H-3	
A	H-1	**C**		C-3
A	C-1	**C**		H-3
B	H-1	**E**	H-4, H-3eq	
B	H-1	**E**		C-4
C	H-1	**B**	H-3	
C	H-1	**B**		C-3
D	H-1	**A**	$H\text{-}6_R$, $H\text{-}6_S$	
D	H-1	**A**		C-6

dis,[29] and show that the sialic acid is *O*-acetylated at either C-7 or C-9 with equal probability in the native LPS preparation. O-Acetylation is a common modification of sialic acids,[30,31] and since it is hypothesized that *O*-acetyl groups predominantly are biosynthesized at C-7 when the exocyclic glyceryl group is modified, followed by transfer to the primary carbon if needed and it is known that they can migrate readily,[32] the present results underscore that the LPS molecule is synthesized under complex biochemical and genetic regulation.

The structure of the O-antigen polysaccharide of *E. coli* O171 determined from the LPS preparations is thus:

→4)-α-Neu5Ac7,9Ac-(2→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→

Analysis of the LPS preparation from *E. coli* O171 by SDS/PAGE showed a ladder pattern due to LPS with different numbers of repeating units. The ^{1}H NMR spectrum of the native LPS showed resonances of approximately equal integral at, inter alia, 2.124 and 2.115 ppm, 2.03 and 2.00 ppm, as well as 1.97 and 1.95 ppm. The two former resonances could be removed by treatment of the material under alkaline conditions and should therefore correspond to *O*-acetyl groups. The alkaline treatment subsequently led to resonances at 2.03 and 2.00 ppm (vide supra) corresponding to the *N*-acetyl groups in the O-antigen polysaccharide. Notably, a small resonance at 2.06 ppm (cf. Fig. 1) was not affected by the chemical modification, and it is suggested that this resonance stems from an *N*-acetylglucosamine residue in the core region. The integrals of the resonances from the methyl groups indicate that the O-antigen polysaccharide contains one equivalent of *O*-acetyl groups per repeating unit and that they are distributed over two positions. Furthermore, the *O*-acetyl groups affect the chemical shifts of the methyl groups of the *N*-acetyl groups.

The ^{1}H NMR spectrum further showed a conspicuous resonance at 5.06 ppm, suggesting it to be derived from O-acetylation at a secondary carbon, since the chemical shift displacement is quite large.[28] Subsequently, $^{1}H,^{1}H$-COSY and $^{1}H,^{1}H$-TOCSY experiments showed spin systems containing four protons that resonate at 5.06, 3.98, 3.66 and 3.48 ppm and at 3.62, 4.07, 4.19 and 4.40 ppm. These chemical shifts are similar to those found for H-7 to H-9 in O-acetylated sialic acid containing capsular polysaccharides from *Neisseria meningiti-*

Sialic acids are often found as terminal sugars in glycolipids and glycoproteins,[33,34] and if the verotoxin-producing *E. coli* O171 infects a host, structural similarities of the terminal sugars may help the bacterium to evade the immune system of the host as a result of molecular mimicry.

3. Experimental

3.1. Bacterial strains and conditions of growth

Shiga toxin producing *E. coli* O171, strain CCUG 36539, 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[14] containing 1% glucose, using a 30 L fermentor (Belach AB) under constant aeration at 37 °C and pH 7.0. A preculture (2 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% (v/v) formaldehyde. After incubation for 16 h at 4 °C, the cells were separated from the medium by continuous-flow centrifugation using a CEPA model LE centrifuge at a cylinder speed of 29,000*g* and a flow of 25 L h^{-1} (Carl Padberg Centrifugenbau, Lahr, Germany). The bacterial mass was then removed from the cylinder, washed once with $NaCl/P_i$ (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 4 °C, 20 min) and finally re-suspended in distilled water.

3.2. Preparation of lipopolysaccharide and O-deacylated lipopolysaccharide

The lipopolysaccharide (LPS) was extracted by the hot phenol/water method.[15] The aqueous phase was dialyzed at 4 °C for 3–5 days against tap water, overnight against distilled water, concentrated under diminished pressure and lyophilized. Contaminating nucleic acids were removed by ultracentrifugation (100,000*g*, 4 h, 4 °C).

O-Deacylation was performed by treatment of 21 mg LPS with 12% aqueous ammonia at 37 °C for 15 h. Subsequent dialysis and lyophilization yielded 16 mg (76%) of O-deacylated LPS.

3.3. Component analysis

The LPS was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 2 h. After reduction with $NaBH_4$ and acetylation, the sample was analyzed by GLC. The absolute configuration of the hexose sugars present in the O-antigen was determined by the derivatization of the sugars as their acetylated (+)-2-butyl glycosides essentially as described.[35]

3.4. GLC analyses

Alditol acetates were separated on a HP-5 column using a temperature program of 180 °C for 1 min, 3 °C min^{-1} to 210 °C, 10 min at 210 °C. Acetylated (+)-2-butyl glycosides were separated on the HP-5 column except for the galactosyl derivatives which were separated on a DB-225 column with a temperature program of 180 °C for 1 min, 3 °C min^{-1} to 210 °C, 1 min at 210 °C, 4 °C min^{-1} to 230 °C, 10 min at 230 °C. Hydrogen was used as carrier gas. The columns were fitted to a Hewlett–Packard model 5890 series II gas chromatograph equipped with a flame ionization detector.

3.5. NMR spectroscopy

NMR spectra of LPS materials in D_2O solutions were recorded at 20 °C using Varian Inova 600 and 800 spectrometers equipped with 5 mm PFG triple-resonance probes, and on a Bruker DRX 500 MHz spectrometer equipped with a 5 mm PFG triple-resonance CryoProbe. Data processing was performed using vendor-supplied software. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-propanoate (TSP, δ_H 0.00) or external 1,4-dioxane in D_2O (δ_C 67.40) as references. The O-deacylated LPS (15 mg) and native LPS (2 mg) were dissolved in 0.7 mL D_2O (pD 5). 2D NMR experiments were used to assign signals, including gHSQC-TOCSY experiments[36] with mixing times of 20 and 50 ms. For inter-residue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment[37] with a mixing time of 50 ms, and an HMBC experiment[38] with a 50 ms delay for the evolution of long-range couplings were used. The chemical shifts were compared to those of the corresponding monosaccharides.[19,39,40]

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