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The structure of the O-specific polysaccharide of *Escherichia coli* O117:K98:H4

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Abstract

The primary structure of the O-antigen of *Escherichia coli* O117 was shown by monosaccharide analysis, methylation analysis, and by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy to be composed of linear pentasaccharide repeating units with the structure:

→3)-α-D-Gal*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→

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1. Introduction

More than 170 different O-groups have been described for the bacterial species *Escherichia coli* [1–3]. Many of these show serological cross-reactions within the species as well as with bacteria belonging to different genera, e.g., *Shigella*, *Salmonella*, and *Klebsiella* [2]. Strains of *E. coli* possessing the O117 antigen have been implicated in septicaemia, and human (neonates and children) and bovine diarrhoea. A case has also been reported [4] in which an *E. coli* O117 strain was sexually transmitted by a woman who displayed symptoms of acute pyelonephritis. The structure of the O-specific polysaccharide of the test strain *E. coli* O117:K98:H4 is reported here. The *E. coli* O117 polysaccharide contains no acidic groups and thus resembles the majority of O-antigens in this series examined to date.

2. Results and discussion

Isolation and composition of the polysaccharide.—*E. coli* O117 bacteria were grown on Mueller–Hinton agar at 37 °C for 20 h, after which the cells were killed with phenol, isolated by centrifugation and washed. The lipopolysaccharide (LPS) was extracted from the lysozyme and ribonuclease-treated cells using a modified version of the phenol extraction procedure [5]. The O-specific polysaccharide (**PS**) was cleaved from lipid A by hydrolysis with 1% acetic acid and was purified by GPC on Sephacryl S-200 SF.

The ^{1}H NMR spectrum of the **PS** (recorded in D_2O at 65 °C; Fig. 1(a)) contained H-1 signals typical for α-linked hexapyranoses at δ 4.975 ($J_{1,2}$ 4.1 Hz) and 4.958 ($J_{1,2}$ 4.0 Hz), a signal for an unresolved doublet at δ 4.906, and two signals for H-1 of β-linked hexapyranoses at δ 4.719 ($J_{1,2}$ 8.2 Hz) and 4.600 ($J_{1,2}$ 7.8 Hz). In addition, signals were observed for the methyl protons of two NAc groups at δ

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2.083 and 2.055, and for H-6 of a deoxy sugar at δ 1.271 ($J_{5,6}$ 6.2 Hz). The ^{13}C NMR data complemented the ^{1}H NMR results and confirmed a pentasaccharide repeating unit for the **PS**, with signals at 105.60, 104.16, 101.29, 100.88, and 99.54 ppm in the anomeric region (95–105 ppm). Signals for carbonyl carbons of the two NAc groups occurred at 176.01 and 175.19 ppm, indicating the absence of a uronic acid, while signals at 53.70 and 49.72 indicated the presence of two C–N bonds. The ^{13}C NMR spectrum of the **PS** (Fig. 1(b)) also contained a signal for the carbon of a methyl group of a 6-deoxy sugar at 17.43 ppm and signals for the methyl carbons of NAc groups at 23.19 and 22.99 ppm.

Hydrolysis of the **PS** with 4 M trifluoroacetic acid followed by GLC–MS examination of the derived alditol acetates showed the presence of GalN, Glc, Gal, and Rha in the ratio 2:1:1:1. Methanolysis of the **PS**, treatment of the products with $NaBH_4$ to effect carboxyl reduction, followed by hydrolysis and GLC–MS examination of the derived alditol acetates gave the same result as before, confirming the absence of a uronic acid in the polymer.

GLC analysis of the derived acetylated (−)-2-octyl glycosides [6] of the **PS** showed the configuration of Rha to be L and that of the other constituents to be D.

The **PS** was incompletely methylated by the Hakomori procedure [7], and the partially methylated polysaccharide was therefore remethylated by the method of Kuhn et al. [8]. GLC and GLC–MS analysis of the partially

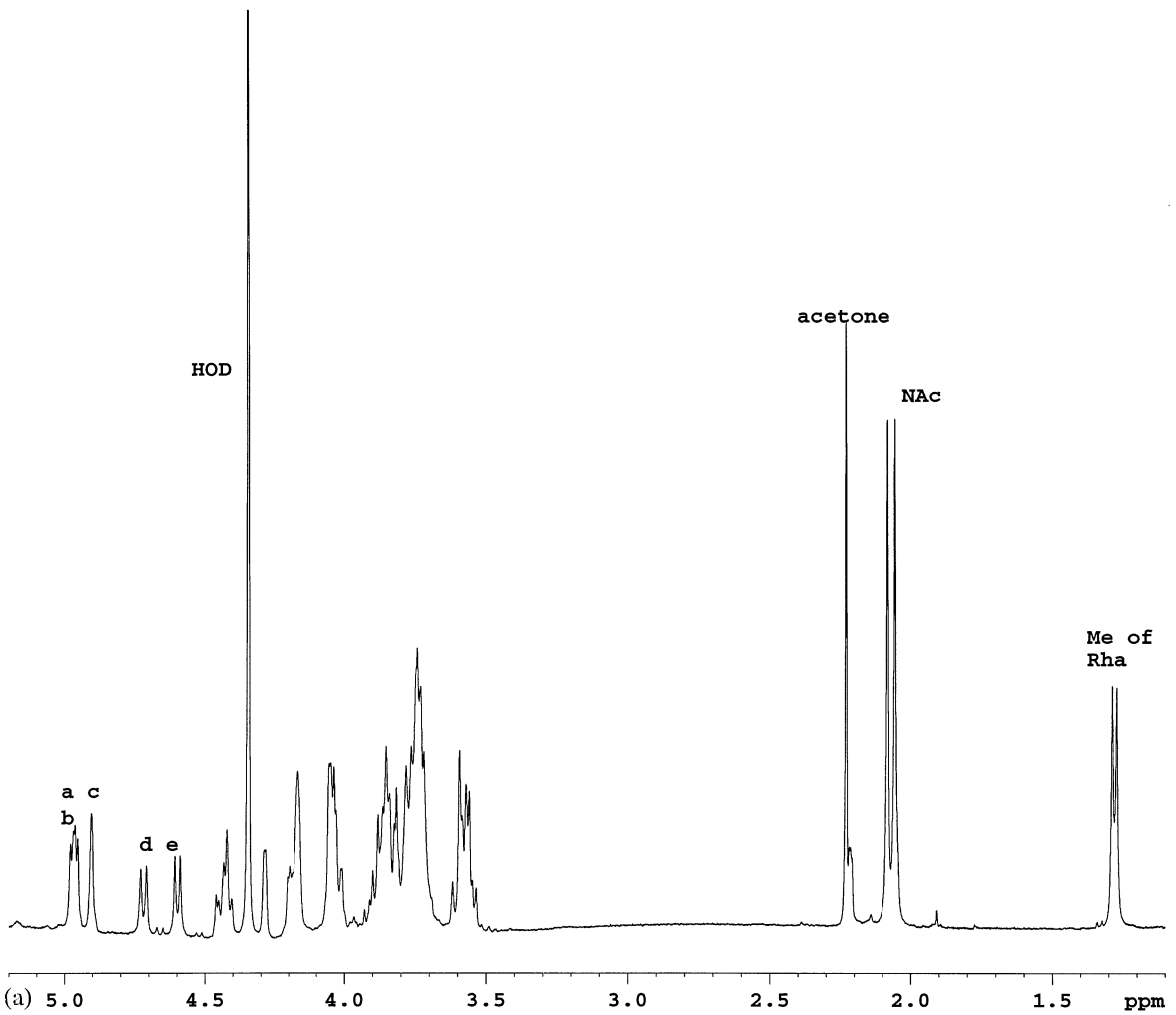

Fig. 1. (a) ^{1}H NMR spectrum of the **PS** in D_2O at 65 °C. For **a**, **b**, **c**, **d**, **e** see text. (b) ^{13}C NMR spectrum of the **PS** in D_2O at 65 °C.

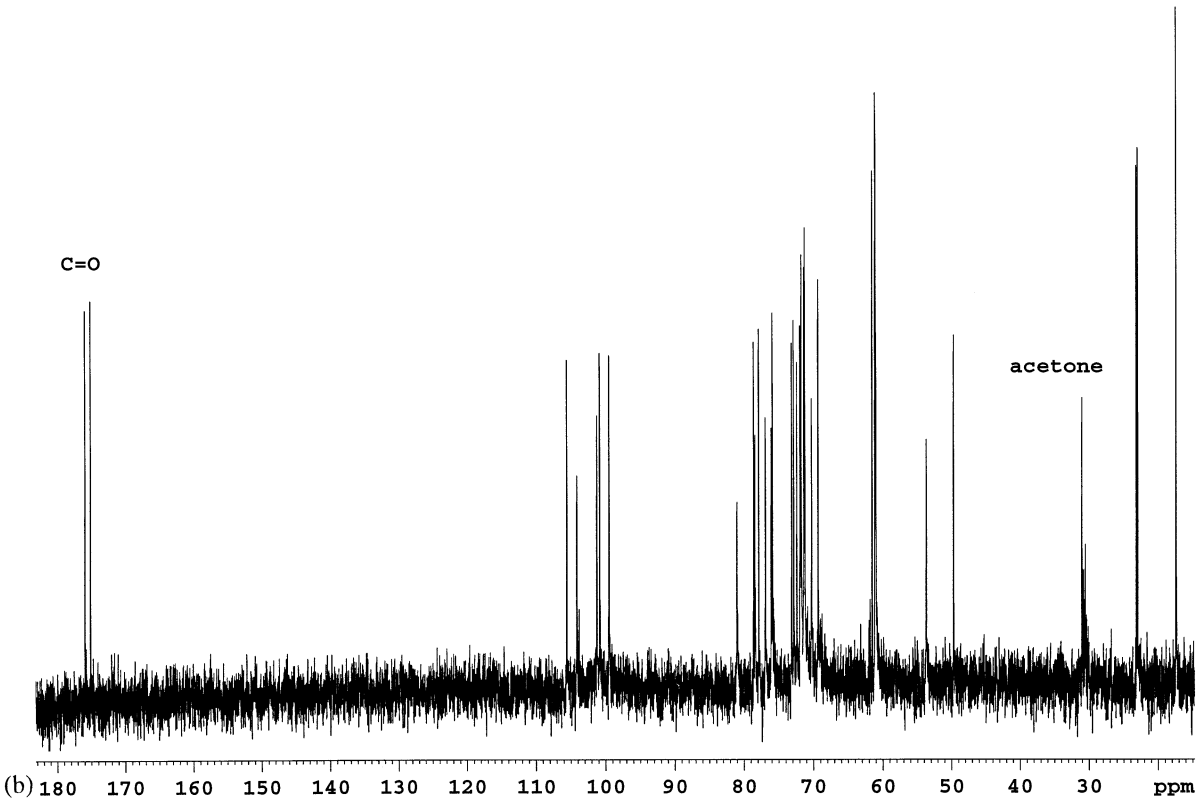

Fig. 1. (*Continued*)

methylated alditol acetates derived from the products of an acid hydrolysate permitted identification of 2-acetamido-2-deoxy-3,6-di-*O*-methylgalactose, 2-acetamido-2-deoxy-4,6-di-*O*-methylgalactose, 2,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,3,6-tri-*O*-methylgalactose. These results indicate the presence of 3-linked GalNAc, 4-linked GalNAc, 3-linked Rha, 4-linked Glc, and 4-linked Gal in a linear pentasaccharide repeating unit.

2D NMR spectroscopy of the ***PS***.—The sequence and linkage positions of the residues in the repeating unit of the **PS** were established by 2D NMR experiments recorded on a solution of the **PS** at 65 °C. The residues were labeled **a**–**e** in order of the decreasing chemical shift of their anomeric protons. Carbon and proton resonances were established from COSY [9], HOHAHA [10], HMQC [11], HMQC-TOCSY [12], HMBC [13], and NOESY [14] experiments. The HMQC contour plot is shown in Fig. 2.

Residue ***a*** *[→3)-α-*D*-Gal*p*NAc]*.—Assignment of ^{1}H resonances for residue **a** was readily achieved by tracing the cross-peaks in the COSY spectrum with confirmation from the HOHAHA spectrum. ^{13}C resonances for all carbons except C-3 and C-6 were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data from the HMQC experiment. Since the shift position of H-3 of residue **a** is very close to those assigned for H-2 of residue **c** and H-5 of residue **b**, the C-3 assignment was made from the HMQC-TOCSY spectrum, which showed clear correlations from H-1 to C-1,2,3,4. The HMQC-TOCSY spectrum also showed correlations in the H-5 track to C-4, C-5, and C-6 of residue **b**, permitting assignment of the C-6 resonance. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-2 of residue **a** and the carbon at 175.19 ppm in the HMBC spectrum, which also showed a correlation between this carbonyl signal and the CH_3 signal at δ 2.083.

Residue ***b*** *[→4)-α-*D*-Glc*p*]*.—As expected for a *gluco* type residue, magnetism relayed well through this spin system. Values were

readily assigned to H-1,2,3 from the cross-peaks in the COSY with confirmation from the HOHAHA spectrum. The HOHAHA spectrum showed magnetism relayed from H-1 to all the other protons of this residue except H-6_b, and from H-5 to all the other protons. Once the H-4 signal had been assigned, the remaining cross-peaks could be followed easily on the COSY spectrum to confirm these assignments. Due to overlap of the H-2 signal with signals from residues **c** and **e**, ^{13}C resonances were assigned with the help of the HMQC-TOCSY spectrum. The H-1 track showed correlations to C-1,2,3,4 and the H-5 track to C-4,5,6.

Residue ***c*** *[→3)-α-*L*-Rha*p*].*—The ^{1}H resonances for residue **c** were readily traced from the cross-peaks in the COSY spectrum and confirmed from the HOHAHA spectrum, which showed correlations from H-6 to the other protons. ^{13}C resonances for C-1,3,5,6 were assigned from the HMQC spectrum by comparison with the ^{1}H assignments. The C-2 and C-4 assignments were obtained from the HMQC-TOCSY spectrum, which showed correlations in the H-6 track to C-2,3,4,5,6 and in the H-1 track to C-1 and C-2.

Residue ***d*** *[→4)-β-*D*-Gal*p*NAc].*—^{1}H resonances for H-1,2,3,4 were assigned from the COSY and HOHAHA spectra, which showed overlap of the H-2 and H-4 resonances. ^{13}C resonances for C-1,2,3,4 were assigned from the HMQC-TOCSY spectrum, which showed correlations from H-1 to these four C signals. These assignments were confirmed from the HMQC spectrum. The H-5 resonance was assigned from the H-1,5 and H-4,5 cross-peaks in the NOESY spectrum. The H-6_a and H-6_b

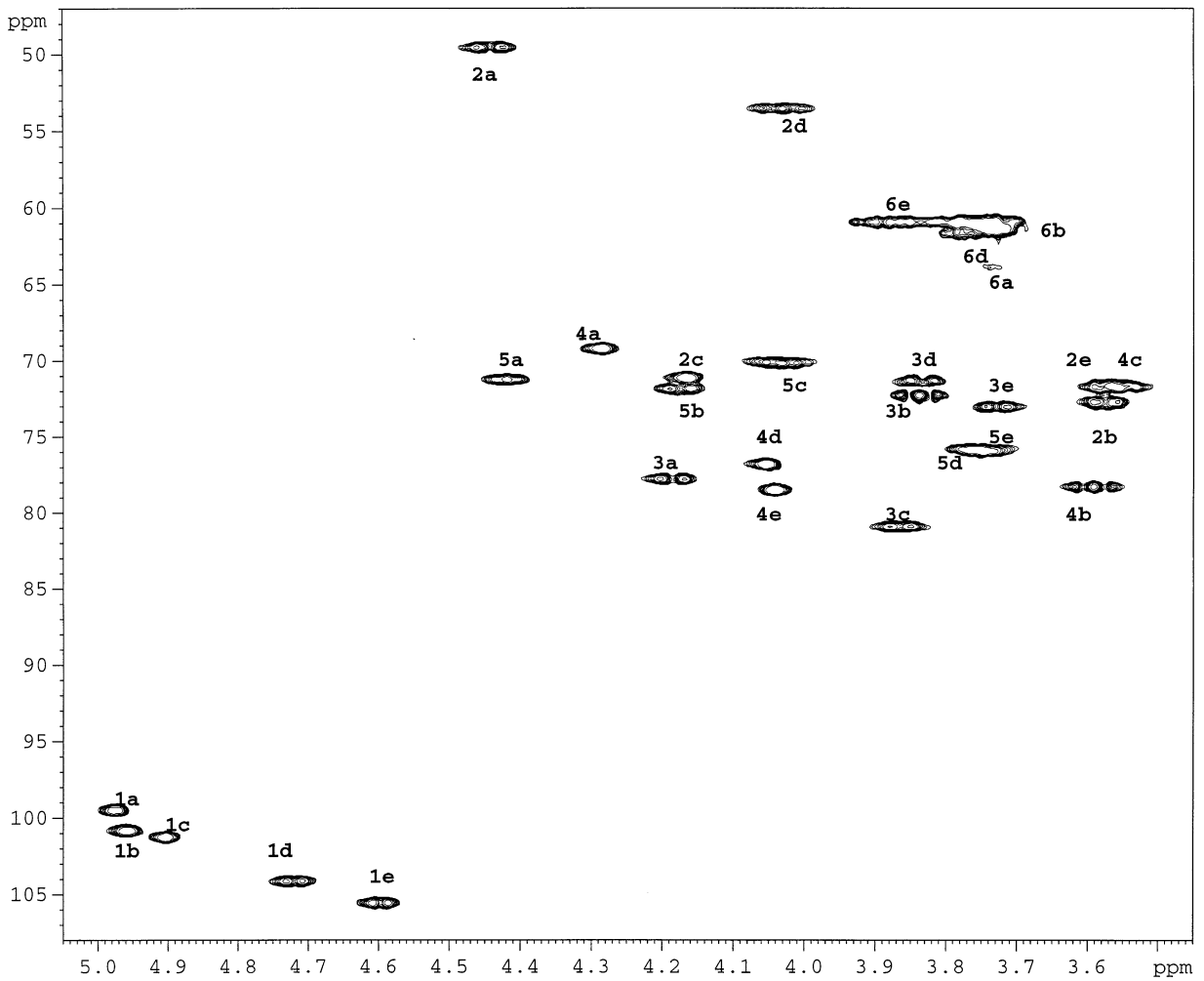

Fig. 2. Partial contour plot of the HMQC experiment on the **PS**. **1a** connotes the cross-peak observed between H-1 and C-1 of residue **a**, etc.

Table 1
NMR data [a] for *E. coli* O117 **PS**

Residue		Proton or carbon								
		1	2	3	4	5	6_a	6_b	NAc CH_3	NAc C=O
→3)-α-D-Gal*p*NAc (**a**)	H	4.975	4.446	4.188	4.289	4.420	3.729	3.729	2.083	175.19
	C	99.54	49.72	**77.89** [b]	69.351	71.37	61.56		23.19	
→4)-α-D-Glc*p* (**b**)	H	4.958	3.576	3.837	3.588	4.178	3.808	3.735		
	C	100.88	72.88	72.41	**78.43** [b]	71.97	61.08			
→3)-α-L-Rha*p* (**c**)	H	4.906	4.167	3.862	3.556	4.033	1.271			
	C	101.29	71.27	**81.03** [b]	71.89	70.30	17.43			
→4)-β-D-Gal*p*NAc (**d**)	H	4.719	4.034	3.837	4.058	3.757	3.900	3.852	2.055	176.01
	C	104.16	53.70	71.37	**76.93** [b]	75.91	61.17		22.99	
→4)-β-D-Gal*p* (**e**)	H	4.600	3.572	3.731	4.041	3.764	3.881	3.820		
	C	105.60	71.76	73.16	**78.61** [b]	76.09	61.01			

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 ppm for ^1H and ^{13}C, respectively.
[b] Linkage carbons are indicated in bold.

resonances were then assigned from the H-5,6 cross-peaks in the COSY spectrum. The C-5 and C-6 resonances were assigned by elimination after all the other C resonances had been allocated. The ^{13}C resonance of the NAc carbonyl signal was assigned from the correlation between H-2 of residue **d** and the carbon at 176.01 ppm in the HMBC spectrum, which also showed a correlation between this carbonyl signal and the CH_3 signal at δ 2.055.

Residue ***e*** *[→4)-β-*D*-Gal*p*].*—^1H resonances for H-1,2,3,4 of residue **e** were assigned from the COSY and HOHAHA spectra. The H-5 resonance was assigned from the H-1,5 and H-4,5 cross-peaks in the NOESY spectrum, and signals could then be assigned to $H\text{-}6_a$ and $H\text{-}6_b$ from the COSY spectrum. ^{13}C resonances for all carbons except C-2 and C-6 could readily be assigned from the HMQC spectrum. The HMQC-TOCSY spectrum showed correlations from H-1 to C-1,2,3,4, and from C-5 to $H\text{-}5,6_a,6_b$. The C-6 signal was identified from the HMBC spectrum, which showed correlations from H-4 to C-5 and C-6 of residue **e**.

The anomeric configurations were assigned by measuring the C-1–H-1 coupling constants obtained from a proton-coupled HMQC experiment [15]. The values obtained were 176.4 Hz for residue **a**, 173.3 Hz for residue **b**, 171.7 Hz for residue **c**, 163.7 Hz for residue **d**, and 163.0 Hz for residue **e**, indicating α configurations for residues **a**, **b** and **c**, and β configurations for residues **d** and **e** [16].

Comparison of the ^1H and ^{13}C chemical shifts for residues **a**–**e** with literature values for methyl glycosides [17–19] identified the residues in the repeating unit as the pyranoses indicated in Table 1. In agreement with the methylation results, the glycosylation sites were established as C-3 for residues **a** and **c** and C-4 for residues **b**, **d**, and **e** by the significant deshielding of these carbon atoms.

Table 2
Two- and three-bond ^1H–^{13}C correlations for **PS**

Residue	Proton	Correlation to
→3)-α-D-Gal*p*NAc (**a**)	H-1	76.93 (**d**; C-4), 77.89 (**a**; C-3), 71.37 (**a**; C-5)
	H-2	175.19 (**a**; NAc C=O)
	H-3	105.60 (**e**; C-1)
	H-5	99.54 (**a**; C-1); 61.56 (**a**; C-6)
→4)-α-D-Glc*p* (**b**)	H-1	78.61 (**e**; C-4), 72.41 (**b**; C-3), 71.97 (**b**; C-5)
	H-4	101.29 (**c**; C-1), 71.97 (**b**; C-5), 61.08 (**b**; C-6)
→3)-α-L-Rha*p* (**c**)	H-1	78.43 (**b**; C-4), 81.03 (**c**; C-3), 70.30 (**c**; C-5)
	H-2	81.03 (**c**; C-3), 71.89 (**c**; C-4)
	H-4	81.03 (**c**; C-3), 17.43 (**c**; C-6)
→4)-β-D-Gal*p*NAc (**d**)	H-1	81.03 (**c**; C-3), 71.37 (**d**; C-3)
	H-3	104.16 (**d**; C-1)
	H-4	99.54 (**a**; C-1)
→4)-β-D-Gal*p* (**e**)	H-1	77.89 (**a**; C-3), 73.16 (**e**; C-3)
	H-2	105.60 (**e**; C-1)
	H-4	100.88 (**b**; C-1), 76.09 (**e**; C-5), 61.01 (**e**; C-6)

Table 3
NOE data for the **PS**

Residue	Proton	NOE to
→3)-α-D-Gal*p*NAc (**a**)	H-1	4.058 (**d**; H-4), 4.446 (**a**; H-2)
	H-5	4.188 (**a**; H-3), 4.289 (**a**; H-4)
→4)-α-D-Glc*p* (**b**)	H-1	4.041 (**e**; H-4); 3.576 (**b**; H-2)
→3)-α-L-Rha*p* (**c**)	H-1	3.588 (**b**; H-4), 4.167 (**c**; H-2)
→4)-β-D-Gal*p*NAc (**d**)	H-1	3.862 (**c**; H-3), 3.837 (**d**; H-3), 3.757 (**d**; H-5)
	H-4	3.757 (**d**; H-5)
→4)-β-D-Gal*p* (**e**)	H-1	4.188 (**a**; H-3), 3.731 (**e**; H-3), 3.764 (**e**; H-5)
	H-4	3.764 (**e**; H-5)

The sequence of the residues in the repeating unit was established from the HMBC and NOESY experiments. The HMBC spectrum showed correlations (Table 2) between C-1 of residue **a** and H-4 of residue **d**, C-1 of residue **b** and H-4 of residue **e**, C-1 of residue **c** and H-4 of residue **b**, C-1 of residue **d** and H-3 of residue **c**, and between C-1 of residue **e** and H-3 of residue **a**. Inter-residue NOEs (listed in Table 3) confirmed these correlations.

3. Conclusions

The combined chemical and NMR data permit the structure of the pentasaccharide repeating unit of the *E. coli* O117 polysaccharide to be written as:

$$\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{NAc}^{\mathbf{a}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc}^{\mathbf{d}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p^{\mathbf{c}}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p^{\mathbf{b}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p^{\mathbf{e}}\text{-}(1\rightarrow$$

The majority of *E. coli* O-antigens are neutral repeating-unit polysaccharides, which can be arranged into groups according to their immune electrophoretic patterns [1,2]. The *E. coli* O117 lipopolysaccharide, classified as a type 1A O-antigen, falls into this majority class. Serological cross-reactivity between the *E. coli* O117 O-antigen and the O-antigens of *E. coli* O50, O76, O101, and O122 has been reported [1,2]. Such cross-reactivity is in some cases due to structural similarity, e.g., the *E. coli* O124 O-specific polysaccharide is identical to the *Shigella dysenteriae* type 3 polysaccharide [20]. Of the O-antigens reported to cross-react with *E. coli* O117, only the structure of the O101 lipopolysaccharide has been published. The O101 polysaccharide shows no first-level structural similarity to that of O117, being composed of a disaccharide repeating unit containing 6-linked-α-D-GlcNAc and 4-linked-α-D-GalNAc residues [21].

4. Experimental

General methods.—Analytical GLC was performed on a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionization detectors and a 3392A recording integrator, with He as carrier gas. A J&W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm, film thickness 0.25 μm) was used for separating alditol acetates and partially methylated alditol acetates (programme I), and acetylated octyl glycosides (programme II). The temperature programmes used were: I, 180 °C for 2 min, then 3 °C/min to 240 °C; II, 180 °C for 2 min, then 2 °C/min to 240 °C. The identification of all derivatives was determined by comparison with authentic standards and confirmed by GLC–MS on a Hewlett–Packard 5988A instrument, using the appropriate column. Spectra were recorded at 70 eV and an ion-source temperature of 200 °C.

Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125 °C. Alditol acetates were prepared by reduction of the products in aq solns of hydrolysates with $NaBH_4$ for 1 h, followed by acetylation with 2:1 Ac_2O–pyridine for 1 h at 100 °C. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h, followed by treatment with $NaBH_4$ in dry MeOH to effect carboxyl reduction. Methylations were carried out using methylsulphinylmethanide [7] and MeI in Me_2SO, followed by a 72 h Kuhn methylation in DMF with Ag_2O and MeI [8].

Preparation of the O117 polysaccharide.—An authentic culture of *E. coli* O117 (culture no. 30w) was propagated on Mueller–Hinton agar (nine trays, 30 × 60 cm, each inoculated with 10 mL liquid culture) at 37 °C for 20 h. The bacterial cells were harvested and mixed with an equal volume of aq 2% phenol. The suspension was stirred (24 h) at 4 °C, dialysed (6000–8000 MW cut-off) against running water for 48 h to remove the phenol, and the slurry was freeze-dried. The dried bacterial cells (18 g) were suspended in 180 mL aq 50 mM Na EDTA containing 0.05% w/v NaN_3 and stirred in a Waring blender at top speed for 1 min. Hen egg-white lysozyme (0.12 g, Sigma Chemical Corp., 50,000 units/mg) was added and the suspension stirred for 16 h at 4 °C, after which the suspension was heated for 10 min at 37 °C and blended again for 3 min. The volume was adjusted to 360 mL with aq 20 mM $MgCl_2$ and bovine pancreas ribonuclease (Sigma Chemical Corp.) was added to a final concentration of 1 μg/mL. After incubating the suspension for 10 min at 37 °C and then for 10 min at 60 °C, the suspension was freeze-dried and the dried cells were extracted using a modified version of the phenol extraction method [5]. The solid material was resuspended in 360 mL H_2O, the suspension heated to 70 °C and added to an equal vol of a 90% w/v soln of phenol, also at 70 °C. The resulting suspension was stirred at 70 °C for 30 min, cooled to room temperature and dialysed against running H_2O (6000–8000 MW cut-off) for 4 days. The solution was filtered to remove solid matter and freeze-dried. The crude polysaccharide was resuspended in 100 mL H_2O and centrifuged at 105,000*g* for 16 h in a Beckman model L8-80M ultracentrifuge. The pellet was collected, resuspended in 50 mL H_2O and centrifuged a second time. The final pellet (5200 mg) was suspended in 1% AcOH (100 mL) and heated at 90 °C for 90 min, after which the solid material was removed by centrifugation (25,000 rpm) and the supernatant was dialysed against running water (3500 MW cut-off) for 24 h and freeze-dried. The crude mixture of O-antigen, KDO and core oligosaccharide (730 mg) was purified by GPC on Sephacryl S-200 SF to yield 500 mg of the O-polysaccharide **PS**.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.230 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 65 °C on a Bruker AMX-400 spectrometer equipped with an X32 computer. The parameters used for 2D experiments were as follows: COSY45 (256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 1644.7 Hz; recycle delay, 1.0 s; unshifted sine-bell filtering in t_1 and t_2). HOHAHA (256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 1644.7 Hz; recycle delay, 1.0 s; mixing time 90.02 ms; shifted sine-squared filtering in t_1 and t_2). NOESY (512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 112 scans per t_1 value; spectral width, 1766.8 Hz; recycle delay, 1.0 s; mixing time 200 ms; shifted sine-squared filtering in t_1 and t_2). HMQC (256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 112 scans per t_1 value; spectral width, 1644.7 Hz in t_2 and 11068.2 Hz in t_1; recycle delay 1.0 s; fixed delay, 3.45 ms; shifted sine-squared filtering in t_1 and t_2). HMQC-TOCSY (512 × 4096 data matrix, zero-filled to 1024 data points in t_1; 80 scans per t_1 value; spectral width, 1644.7 Hz in t_2 and 11068.2 Hz in t_1; recycle delay, 1.0 s; fixed delay, 3.45 ms; mixing time, 90.02 ms; shifted sine-squared filtering in t_1 and t_2). HMBC (256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 1644.7 Hz in t_2 and 22640.3 Hz in t_1; recycle delay, 1.0 s; fixed delay, 3.45 ms; shifted sine-squared filtering in t_1 and t_2). HMQC without ^1H decoupling (256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 152 scans per t_1 value; spectral width, 2403.8 Hz in t_2 and 14086.8 Hz in t_1; recycle delay, 1.0 s; fixed delay, 3.45 ms; shifted sine-squared filtering in t_1 and t_2).

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