Structure of the O-polysaccharide of *Escherichia coli* O60

A. V. Perepelov, O. I. Naumenko, S. N. Senchenkova, A. S. Shashkov, A. O. Chizhov, and Yu. A. Knirel★

N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences,
47 Leninsky prosp., 119991 Moscow, Russian Federation.
Fax: +7 (499) 137 6148. E-mail: yknirel@gmail.com

Structure of the O-polysaccharide (O-antigen) of *Escherichia coli* O60 was studied by sugar analysis, partial solvolysis with CF_3CO_2H, and 1D and 2D ^1H and ^{13}C NMR spectroscopy. The O-polysaccharide was found to consist of D-galactose and L-rhamnose. The structure of its branched tetrasaccharide repeating unit was established, which is unique among known bacterial polysaccharide structures.

Key words: *Escherichia coli*, bacterial polysaccharide structure, O-antigen, lipopolysaccharide, solvolysis.

Escherichia coli is a clonal bacterial species that contains both commensal and pathogenic strains. The latter include causative agents of diarrhea (escherichiosis) and more serious diseases, such as enterocolitis, hemorrhagic colitis, and hemolytic-uremic syndrome. *E. coli* is one of the most serologically heterogeneous bacterial species, and, based on O-antigens, its strains are classified into more than 180 O-serogroups (https://www.ssidiagnostica.com/e-coli-o-antisera/?cid=34). From the chemical point of view, the O-antigen or O-specific polysaccharide represents a polysaccharide chain of the lipopolysaccharide, which is located on the outer leaflet of the outer membrane of the cell wall of *E. coli* and other gram-negative bacteria.[1] Structural diversity of the O-polysaccharides, which are the most structurally variable cell component, helps adaptation of bacteria in various ecological niches and is considered as one of the virulence factors, which enables pathogenic strains to escape the protective action of the adaptive immunity.

Determination of O-polysaccharide structures as a chemical basis for classification of *E. coli* began more than 50 years ago but has not been completed yet owing to a high diversity of the O-antigen forms (http://nevyn.organ.su.se/ECODAB/). In this work, we established the O-polysaccharide structure of *E. coli* O60, which is one of a few that have not been elucidated earlier.

Results and Discussion

A high-molecular weight O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from bacterial cells by the phenol-water extraction. Sugar analysis by GC of the alditol acetates derived after full acid hydrolysis of the O-polysaccharide revealed rhamnose (Rha) and galactose (Gal) in the ratio of ~1 : 1. Gas chromatography analysis of the acetylated (*S*)-2-octyl glycosides demonstrated the D configuration of Gal and the L configuration of Rha.

The ^{13}C NMR spectrum of the O-polysaccharide (Fig. 1) showed signals for four anomeric carbons in the region δ 100.1—104.3, two $\underline{C}H_3$—C groups ($C_{Rha}(6)$) at δ 17.6 and 17.9, two $HO\underline{C}H_2$—C groups ($C_{Gal}(6)$) at δ 62.0 and 62.8, and 16 oxygen-bearing non-anomeric sugar ring carbons in the region δ 69.7—79.5 (Table 1).

The ^1H NMR spectrum of the O-polysaccharide contained signals for four anomeric protons at δ 4.48—5.31, two methyl groups ($H_{Rha}(6)$) at δ 1.27 and 1.34, and other sugar protons in the region δ 3.48—4.27. Therefore, the O-polysaccharide is composed of tetrasaccharide O-units containing two residues each of D-Gal (units **A** and **D**) and L-Rha (units **B** and **C**).

Signals in the ^1H and ^{13}C NMR spectra of the O-polysaccharide were assigned using 2D $^1H,^1H$ COSY, $^1H,^1H$ TOCSY, and $^1H,^{13}C$ HSQC experiments (see Table 1). Based on intra-residue H,H and H,C correlations and $^3J_{H,H}$ coupling constant values, spin systems were assigned to units **A**—**D**, all being in the pyranose form. A position at δ 70.9 of the signals for C(5) indicated that units **B** and **C** are α-linked (*cf.* Ref. 2: δ 69.5 and 73.2 for α- and β-Rha*p*, respectively). The chemical shift for the C(5) atom of δ 73.3 showed that unit **D** is α-linked, whereas that of δ 76.7 indicated that unit **A** is β-linked (*cf.* Ref. 2: δ 71.7 and 76.3 for α- and β-Gal*p*, respectively).

Positions of substitution of the monosaccharides were revealed by downfield shifts of the signals for the C(2) and C(3) atoms of unit **B**, the C(4) atom of unit **A**, and the C(2) atom of unit **C** to δ 76.3—79.5 as compared with their positions at δ 70.0—72.1 in the corresponding non-substituted monosaccharides. The C(2), C(3), C(4), and C(6) chemical shifts of unit **D** differed from those of α-D-Gal*p*

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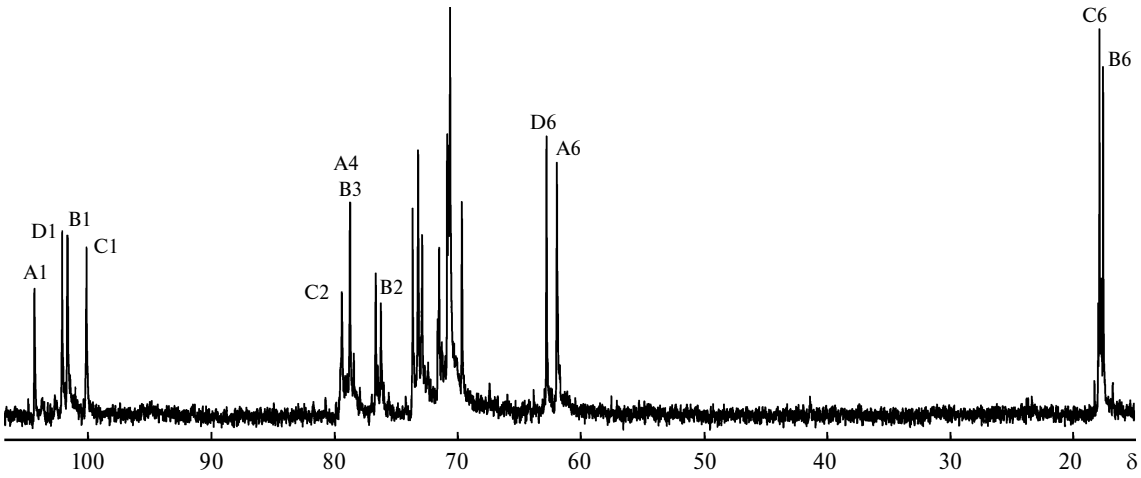

Fig. 1. ^{13}C NMR spectrum of the O-polysaccharide of *E. coli* O60. Numbers refer to carbon atoms in the sugar residues denoted by letters as shown in Table 1.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ) of the O-polysaccharide of *E. coli* O60*

Monosaccharide residue	H(1) C(1)	H(2) C(2)	H(3) C(3)	H(4) C(4)	H(5) C(5)	H(6a), H(6b) C(6)
→4)-β-D-Gal*p*-(1→	4.48	3.58	3.78	4.01	3.76	3.76, 3.76
(**A**)	*104.3*	*71.6*	*72.9*	*78.9*	*76.7*	*62.0*
→2,3)-α-L-Rha*p*-(1→	4.84	4.27	4.02	3.71	4.17	1.27
(**B**)	*101.6*	*76.3*	*78.8*	*73.3*	*70.9*	*17.6*
→2)-α-L-Rha*p*-(1→	5.28	4.21	3.84	3.48	3.86	1.34
(**C**)	*100.1*	*79.5*	*70.7*	*73.7*	*70.9*	*17.9*
α-D-Gal*p*-(1→	5.31	3.86	3.83	3.99	3.96	3.73, 3.80
(**D**)	*102.2*	*69.7*	*70.8*	*70.7*	*73.3*	*62.8*

* ^{13}C NMR chemical shifts are italicized.

insignificantly. Therefore, the O-polysaccharide has a branched repeating unit with residue **B** at the branching point and terminal residue **D** in the side chain.

The 2D $^{1}H,^{1}H$ ROESY spectrum of the O-polysaccharide showed the following correlations between anomeric protons and protons at the linkage carbons: $H_{\mathbf{D}}(1)/H_{\mathbf{B}}(3)$; $H_{\mathbf{B}}(1)/H_{\mathbf{A}}(4)$; $H_{\mathbf{A}}(1)/H_{\mathbf{C}}(2)$, and $H_{\mathbf{C}}(1)/H_{\mathbf{B}}(2)$ at δ 5.31/4.02, 4.84/4.01, 4.48/4.21, and 5.28/4.27, respectively. The $^{1}H,^{13}C$ HMBC spectrum showed correlations between anomeric protons and linkage carbons (Fig. 2) and *vice versa*. These data confirmed the glycosylation pattern and defined the monosaccharide sequence in the repeating unit.

To confirm the structure, the O-polysaccharide was subjected to partial solvolysis with CF_3CO_2H[3] to give a mixture of products. They were separated by gel-permeation chromatography on Fractogel TSK HW-40S to give four oligosaccharide fractions (I—IV) with fractions I and IV being the dominant ones. The fractions were analyzed by positive ion mode high-resolution electrospray ionization mass spectrometry (Table 2) and NMR spectroscopy.

The data obtained showed that fraction II contained a mixture of $Hex_2$6dHex_2 tetrasaccharides and fraction I included a mixture of higher oligosaccharides form heptasaccharides to nonasaccharides (see Table 2). Fraction III contained a $Hex_1$6dHex_2 trisaccharide with an $[M + Na]^+$ ion peak at *m/z* 495.1670 in the mass spectrum. The 2D NMR spectra showed that the main component of this fraction was a β-Gal*p*-(1→2)-α-Rha*p*-(1→2)-Rha trisaccharide (δ_H 4.48, 5.10, and 5.23; δ_C 103.6, 101.7, and 94.0 for linked β-Gal, linked α-Rha, and α-Rha at the reducing end, respectively).

The mass spectrum of fraction IV showed a major $[M + Na]^+$ ion peak at *m/z* 349.1101, which corresponded to a $Hex_1$6dHex_1 disaccharide. The ^{1}H NMR spectrum showed the presence of a linked β-Gal residue (H(1) at δ_H 4.46, C(1) at δ_C 103.7) and a linked α-Gal residue (H(1) at δ_H 5.28 and C(1) at δ_C 102.0) in the ratio ~1.7 : 1, respectively. Therefore, it was concluded that fraction IV contained a mixture of β-Gal*p*-(1→2)-Rha and α-Gal*p*-(1→3)-Rha disaccharides. This finding was in agreement with the known higher stability towards solvolytic cleavage

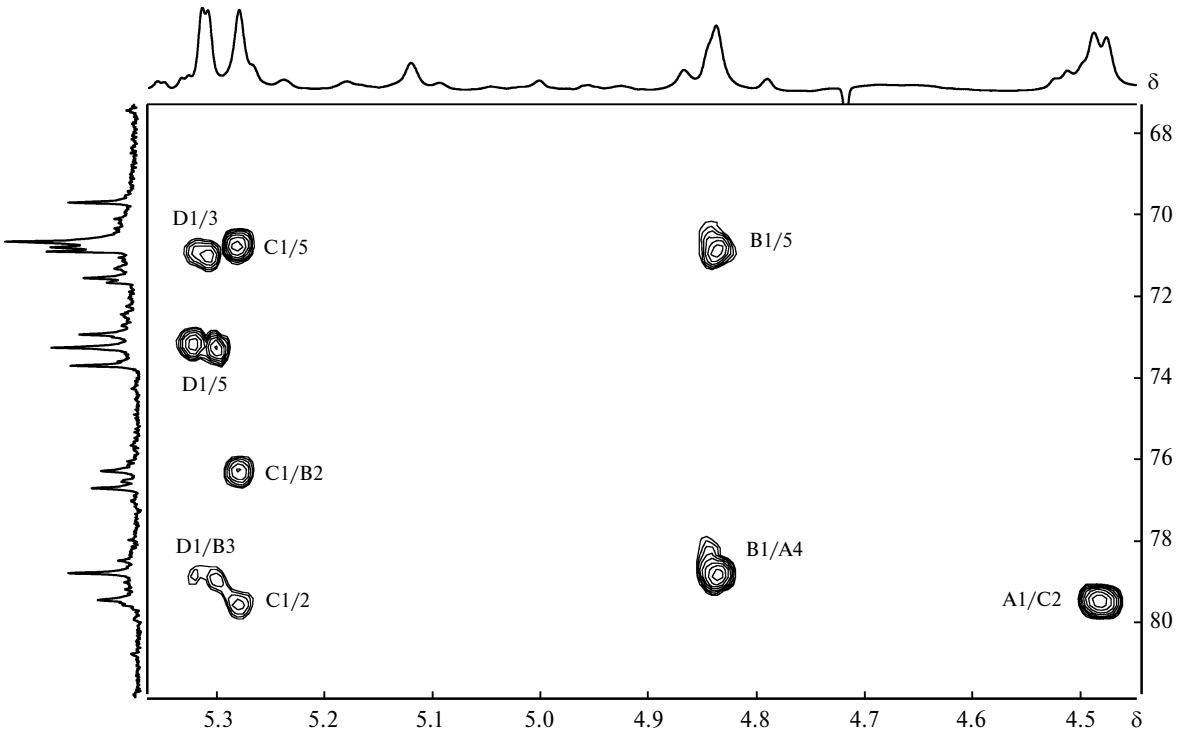

Fig. 2. Fragment of a $^{1}H,^{13}C$ HMBC spectrum of the O-polysaccharide of *E. coli* O60. The corresponding fragments of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals before and after oblique stroke refer to protons and carbon atoms, respectively, in sugar residues denoted by letters as shown in Table 1.

of the glycosidic linkage of hexoses as compared with the linkage of 6-deoxyhexoses.[3]

Therefore, the results of solvolysis were in agreement with the O-polysaccharide structure established by sugar analysis and NMR spectroscopy. To our knowledge, the O-polysaccharide structure of *E. coli* O60 shown in Fig. 3 is unique among known bacterial polysaccharide structures.

Table 2. High-resolution electrospray ionization mass spectral data of oligosaccharides derived from the O-polysaccharide of *E. coli* O60 by solvolysis with CF_3CO_2H

Composition of oligosaccharide*	Molecular weight/Da	$[M + Na]^+$ ion peak at m/z	
		experimental	calculated
	Fraction IV		
$Hex_1$6dHex_1	326.1213	349.1101	349.1105
	Fraction III		
$Hex_1$6dHex_2	472.1792	495.1670	495.1684
	Fraction II		
$Hex_2$6dHex_2	634.2320	657.2182	657.2213
	Fraction I		
$Hex_2$6dHex_5	1072.4058	1095.3927	1095.3950
$Hex_3$6dHex_4	1088.4007	1111.3877	1111.3899
$Hex_4$6dHex_3	1104.3956	1127.3777	1127.3848
$Hex_3$6dHex_5	1234.4586	1257.4455	1257.4478
$Hex_4$6dHex_4	1250.4535	1273.4382	1273.4427
$Hex_3$6dHex_6	1380.5165	1403.5021	1403.5057
$Hex_4$6dHex_5	1396.5114	1419.4972	1419.5006
$Hex_5$6dHex_4	1412.5063	1435.4862	1435.4955

* Hex and 6dHex indicate hexose and 6-deoxyhexose, respectively.

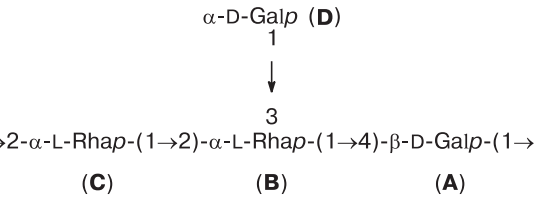

Fig. 3. Structure of the O-polysaccharide of *E. coli* O60.

The O-polysaccharide structure established does not match the content of the O-antigen gene cluster at the typical location on the *E. coli* chromosome.[4] Thus, the gene cluster contains *manB*, *manC* and *gmd* genes for synthesis of biosynthetic precursors of D-mannose and L-fucose, which are not the components of the O-polysaccharide of *E. coli* O60, and no genes for synthesis of a biosynthetic precursor of L-rhamnose, which is a component of the O-polysaccharide, are present in the cluster. Hence, the functional gene cluster for biosynthesis of the O-antigen of *E. coli* O60 is located elsewhere in the genome; it remains to be found and characterized. A similar situation, namely the presence of a non-functional gene cluster at the typical location and a functional gene cluster elsewhere on the chromosome, has been reported by us for the O-antigen of *E. coli* O62.[5]

Experimental

E. coli O60 type strain was obtained from the Institute of Medical and Veterinary Science (Adelaide, Australia). Bacteria were grown to late log phase in 8 L of Luria—Bertani broth using a 10-L BIOSTAT C-10 fermenter (B. Braun Biotech Int., Germany) under constant aeration at 37 °C and pH 7.0. Bacterial cells were washed and dried as earlier described.[6]

A lipopolysaccharide sample was isolated from bacterial cells in 6.9% yield by phenol-water extraction,[7] the crude extract was dialyzed without separation of the layers and freed from nucleic acids and proteins by treatment with 50% aqueous CCl_3CO_2H at 4 °C to pH 2. The precipitate was removed by centrifugation, and the supernatant was dialyzed and lyophilized.

Mild acid degradation (100 °C, 1 h) of the lipopolysaccharide (106 mg) was performed with 2% aqueous AcOH. The precipitate was removed by centrifugation (13 000 *g*, 20 min), and the supernatant was fractionated by gel-permeation chromatography on a column (56×2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 0.05 *M* pyridinium acetate buffer (pH 5.5); chromatography was monitored with a differential refractometer (Knauer, Germany). The O-polysaccharide yield was 52% of the lipopolysaccharide weight.

An O-polysaccharide sample (1 mg) was hydrolyzed (120 °C, 2 h) with 2 *M* CF_3CO_2H. Monosaccharides were identified by GC of the alditol acetates on a Maestro (Agilent 7820) gas chromatograph (Interlab, Russia) equipped with a HP-5ms column (0.32 mm×30 m) using a temperature program of 160 °C (1 min) to 290 °C at a heating rate of 7 deg min^{-1}. The absolute configurations of the monosaccharides were determined by GC of the acetylated (*S*)-2-octyl glycosides as earlier described.[8]

An O-polysaccharide sample (12 mg) was treated with anhydrous CF_3CO_2H (0.2 mL) at 90 °C for 1 h. After evaporation of the volatiles, the reaction products were dissolved in water and fractionated by gel-permeation chromatography on a column (85×1.6 cm) of Fractogel TSK HW-40S in 1% AcOH to give oligosaccharide fractions I—IV (2.6, 1.8, 1.0, and 2.7 mg, respectively) and a monosaccharide fraction (1.5 mg).

For measuring NMR spectra, an O-polysaccharide sample was freeze-dried from 99.9% D_2O and dissolved in 99.95% D_2O. The NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer (Germany) at 20 °C using sodium 3-(trimethylsilyl) propanoate-2,2,3,3-d_4 (δ_H 0.0, δ_C −1.6) as an internal reference. The 2D NMR spectra were obtained using standard Bruker software; TopSpin 2.1 program (Bruker) was used to acquire and process the NMR data. A mixing time of 100 and 150 ms was used in the TOCSY and ROESY experiments, respectively. A 60-ms delay was used in the $^1H,^{13}C$ HMBC experiment for development of multi-bond correlations.

Positive ion mode high-resolution electrospray ionization mass spectra were measured on a Bruker micrOTOF II instrument. Internal calibration was done with Electrospray Calibrant Solution (Fluka). Samples (~50 ng μL^{-1}) were dissolved in a 1 : 1 (v/v) H_2O—MeCN mixture and sprayed at a flow rate of 3 $\mu L\ min^{-1}$ using nitrogen as the nebulizing gas (4 L min^{-1}). The capillary entrance voltage was −3000 V, exit voltage was 150 V, and interface temperature was 180 °C.

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