CARBOHYDRATE RESEARCH

ELSEVIER Carbohydrate Research 277 (1995) 283–290

Structure of the O-specific side chain of the *Escherichia coli* O128 lipopolysaccharide

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Received 8 February 1995; accepted 5 May 1995

Abstract

The O-specific polysaccharide isolated from *Escherichia coli* O128 lipopolysaccharide contains D-galactose, L-fucose, and 2-acetamido-2-deoxy-D-galactose in the molar ratios 2:1:2. The primary structure of the O-specific polysaccharide from *E. coli* was established by compositional analysis, methylation analysis, together with ^{1}H and ^{13}C NMR spectroscopy including two-dimensional shift-correlated and one-dimensional NOE spectroscopy. The polysaccharide moiety was found to consist of a tetrasaccharide backbone containing D-galactose and 2-acetamido-2-deoxy-D-galactose, with L-fucose as a side chain in a branched pentasaccharide repeating unit having the following structure:

→ 3)-β-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 6)-β-D-Gal*p*-(1 →
2
↑
1
α-L-Fuc*p*

Keywords: *Escherichia coli*; *E. coli* O128; O-Antigen; Lipopolysaccharide; NMR

1. Introduction

Escherichia coli is a Gram-negative and complex group of bacteria which include a large number of strains that differ in pathogenic potential [1,2]. The most pathogenic

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SSDI 0008-6215(95)00215-4

role of *E. coli* is to produce diarrhoeal diseases. The enteropathogenic *E. coli* strains (EPEC) are known to be associated with infantile diarrhoea which is one of the major causes of illness and death among children in developing countries. The *E. coli* species consists of a large number of more or less closely related serovars. They can be differentiated in many ways and by many techniques, but until now the most practical and useful procedure has been based on the nature of O-antigens, i.e., the O-specific polysaccharide part of the lipopolysaccharide (LPS) which is the major outer-membrane component of the Gram-negative bacteria [3]. A thorough and precise knowledge of the O-antigens of *E. coli* is clearly important. At present 173 different O-serotypes of *E. coli* are known [4], and several chemical and serological studies have been made on the O-specific polysaccharide in order to understand the basis of serological specificity and the complex pathogenicity of *E. coli* [5]. In this communication, we present the results of chemical investigations carried out on the O-specific polysaccharide isolated from an enteropathogenic strain of *E. coli* O128.

2. Results and discussion

Isolation and characterisation of the O-specific polysaccharide.—The LPS was extracted from *E. coli* O128 cells, using the hot aqueous phenol method and ultracentrifugation of the aqueous phase [6]. The sediment was found to contain short-chain LPS and R-LPS. The supernatant solution contained long-chain LPS which was isolated by fractional precipitation with cetyltrimethylammonium bromide [6,7]. This LPS with a longer O-specific polysaccharide chain was used for structural analysis. Partial hydrolysis of the long-chain LPS with hot acetic acid yielded an insoluble lipid A (15% yield), and gel-permeation chromatography of the water-soluble products on Sephadex G-50 gave an O-polysaccharide (48% yield, K_{av} 0.05), a core oligosaccharide (10% yield, K_{av} 0.69), and a fraction containing 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and phosphate (4% yield, K_{av} 0.92).

Structural studies of the O-specific polysaccharide.—Microanalysis of the O128 polysaccharide gave C, 46.5; H, 6.5; N, 3.2. Paper chromatography of the polysaccharide hydrolysate as well as GLC analysis of the alditol acetates showed that the polysaccharide from *E. coli* O128 consists of galactose, fucose, and 2-amino-2-deoxygalactose. The quantitative determinations of the constituent sugars showed that they are present in the molar ratios 2:1:2. The optical rotation values of the sugar monomers isolated from the polysaccharide hydrolysate by preparative paper chromatography enables assignment of the D configuration to the galactose and 2-amino-2-deoxygalactose, and L to the fucose residues.

The polysaccharide was methylated with NaOH–MeI in Me_2SO as described previously [9]. Alditol acetates obtained from the hydrolysate of the methylated polysaccharide revealed (GLC-MS) derivatives of 2,3,6-tri-*O*-methylgalactose, 3,4-di-*O*-methylgalactose, 2-deoxy-4,6-di-*O*-methyl-2(*N*-methylacetamido)galactose, and 2,3,4-tri-*O*-methylfucose. Hence, the polysaccharide has a branched structure: the fucopyranosyl group occupies the nonreducing terminus of the side chain, the one galactopyranoside residue substituted at positions O-2 and O-6 represents a branch point, the other

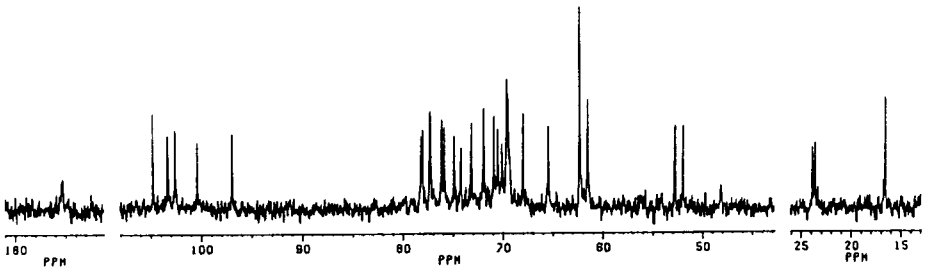

Fig. 1. 75-MHz ^{13}C NMR spectrum of the *E. coli* O128 polysaccharide.

galactopyranoside residue is substituted at O-4, while the galactosamine residues in their pyranosidic form are substituted at O-3.

Smith degradation of the polysaccharide [9] destroyed all the sugar constituents, with the exception of 2-acetamido-2-deoxy-D-galactose.

The ^{13}C NMR spectrum (Fig. 1) of the O-specific polysaccharide showed the following characteristic signals: five major signals in the pyranose anomeric carbon region (104.7–96.9 ppm), two signals for deoxy amino-substituted carbon atoms (52.8 and 51.9 ppm), one signal for the methyl carbon of a 6-deoxyhexose at 16.5 ppm, signals for two acetamido groups (CH_3 at 23.8 and 23.6 ppm, and CO at 175.3 and 175.2 ppm). An Attached Proton Test (APT) spectrum [10] indicated the presence of one substituted hydroxymethyl group (69.3 ppm) and three unsubstituted hydroxymethyl groups (62.4–61.5 ppm). No signals typical of furanoses were observed in the ^{13}C NMR spectrum [11]. A coupled ^{13}C NMR spectrum indicated the presence of two α- and three β-anomeric linkages [12].

The ^1H NMR spectrum of the polysaccharide (Fig. 2) showed the major signals for five anomeric protons at δ 5.24 to 4.53, three of them having coupling constants ($J_{1,2}$ 7.5–8.0 Hz) typical of sugars with *β-gluco/galacto* configuration and two signals for sugars with the *α-gluco/galacto* configuration ($J_{1,2}$ 4 Hz). The signal at δ 1.21 (doublets with $J_{1,2}$ 6 Hz) was assigned to the methyl group of the 6-deoxy sugar. The signal at δ 2.04 can be assigned to the *N*-acetyl methyl group of the hexosamines, and the signals at δ 4.2–3.6 correspond to other protons of the constituent monosaccharides of the O-specific polysaccharide.

Both ^{13}C and ^1H spectral data (Table 1) suggested that the O-specific polysaccharide is composed of pentasaccharide repeating units which are consistent with the chemical data.

Detailed two-dimensional NMR experiments were used to assign the primary structure of the O128 polysaccharide. Homonuclear spin decoupling experiments in a modified differential mode [13], two-dimensional homonuclear COSY, and one- and two-step H-relayed two-dimensional coherence transfer COSY [14,15] allowed complete assignment of the ^1H NMR spectrum (Table 1). Analysis of chemical shifts [16] and coupling constants [17] revealed that the repeating unit of the O128 polysaccharide contains sugars with galactopyranosidic configuration, in the form of α- and β-galactopyranose residues and 2-acetamido-2-deoxy-β-galactopyranose residues, together with an α-fucopyranose residue.

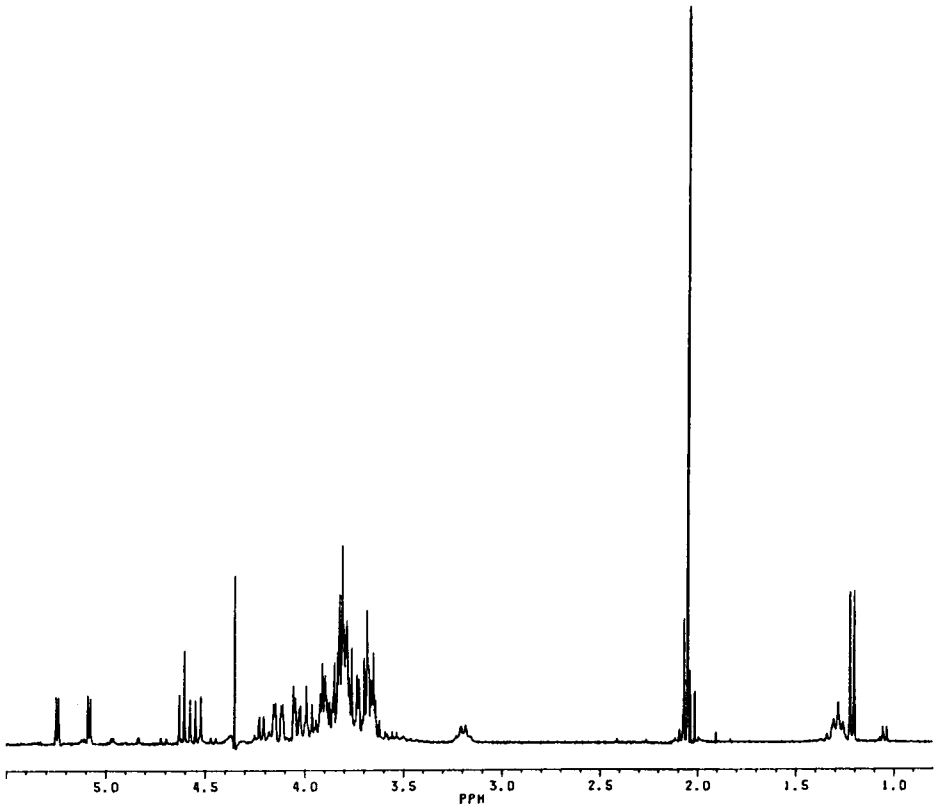

Fig. 2. One-dimensional 300-MHz ^{1}H NMR spectrum of the *E. coli* O128 polysaccharide.

The sequence and type of substitution of the constituent monosaccharide residues of the polysaccharide were unequivocally determined by a series of 1D NOE experiments (Table 2). Measurements were carried out in the difference mode, and the transglycosidic NOEs were observed between the anomeric and aglyconic protons of the contiguous residues, thus establishing the sequence of the sugar residues.

Preirradiation of H-1 of Gal*p*NAc residue (unit **A** as described in the structural diagram below) resulted in a considerable NOE response on H-4 of one Gal*p* residue (unit **B**) at 4.11 ppm. In turn, in the spectrum obtained with preirradiation of H-1 of unit **B** at 5.08 ppm, an NOE of H-3 and H-4 of the Gal*p*NAc residue (unit **C**) at 3.81 and 4.15 ppm, respectively, was observed. Preirradiation of H-1 of unit **C** at 4.59 ppm caused an NOE effect on H-6 of unit **D** (Gal*p* residue) and preirradiation of H-1 of unit **D** at 4.61 ppm showed an NOE at H-3 of unit **A** at 3.90 ppm. These results indicated that the main chain, or the backbone, of the polysaccharide is composed of tetrasaccharide repeating units for which units **A** and **C** (Gal*p*NAc residues) are substituted at position 3, but unit **B** (one Gal*p* residue) is substituted at position 4 and unit **D** (the other Gal*p* residue) is substituted at position 6. Besides the above-mentioned responses,

Table 1
^{1}H and ^{13}C NMR data [a] for the *E. coli* O128 polysaccharide

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ	Glycosylation effects
→ 3)-β-D-Gal*p*NAc-(1 →	H-1	4.53	$J_{1,2}$	8	C-1	104.7	
(**A**)	H-2	3.99 [b]	$J_{2,3}$	9	C-2	52.8 [c]	
	H-3	3.90	$J_{3,4}$	3.5	C-3	78.2	
	H-4	4.05	$J_{4,5}$	< 2	C-4	69.6	+0.5
	H-5	3.68			C-5	75.9	
	H-6a, 6b	~ 3.80			C-6	62.4	
→ 4)-α-D-Gal*p*-(1 →	H-1	5.08	$J_{1,2}$	4	C-1	96.9	+3.4
(**B**)	H-2	3.68	$J_{2,3}$	10	C-2	69.6	
	H-3	3.87	$J_{3,4}$	3	C-3	70.5	
	H-4	4.11	$J_{4,5}$	< 2	C-4	78.0	
	H-5	3.82			C-5	71.9	
					C-6	61.5	−0.9
→ 3)-β-D-Gal*p*NAc-(1 →	H-1	4.59	$J_{1,2}$	8	C-1	102.6	
(**C**)	H-2	4.03 [b]	$J_{2,3}$	9.5	C-2	51.9 [c]	
	H-3	3.81	$J_{3,4}$	3	C-3	77.2	
	H-4	4.15	$J_{4,5}$	< 2	C-4	65.4	−3.7
	H-5	3.66			C-5	76.1	
	H-6a, 6b	~ 3.8			C-6	62.4	
→ 6)-β-D-Gal*p*-(1 →	H-1	4.61	$J_{1,2}$	7.5	C-1	103.3	
(**D**)	H-2	3.65	$J_{2,3}$	9.5	C-2	77.3	
	H-3	3.81	$J_{3,4}$	4	C-3	74.9	+0.8
	H-4	3.90	$J_{4,5}$	< 2	C-4	70.0	
	H-5	3.77			C-5	74.2	
	H-6a, 6b	3.79			C-6	69.3	
α-L-Fuc*p*-(1 →	H-1	5.24	$J_{1,2}$	4	C-1	100.4	+7.3
(**E**)	H-2	3.78	$J_{2,3}$	8	C-2	69.5	
	H-3	3.66	$J_{3,4}$	3.5	C-3	70.9	
	H-4	3.73	$J_{4,5}$	< 2	C-4	73.1	
	H-5	4.22	$J_{5,6}$	6.5	C-5	68.0	
	H-6	1.21			C-6	16.5	

[a] Recorded in D_2O, with acetone (δ ^{1}H 2.225; δ ^{13}C 31.45) as internal standard.
[b] Signal for CH_3CO at δ 2.045.
[c] Signals for CH_3CONH at δ 23.6 or 23.8 and 175.25 or 175.35.

intraresidue NOEs were observed between H-1 and H-2 of each of the residues and, in addition, units **A**, **C**, and **D** showed NOEs to their respective H-3 and H-5 protons, thus clearly establishing their β-anomeric configuration. Irradiation of H-1 of the L-fucose residue (unit **E**) showed a considerable NOE on H-2 of unit **D**, thus confirming that unit **E** was directly attached to the main chain at position 2 of residue **D**. Assignments of ^{13}C NMR spectral lines of the *E. coli* O128 polysaccharide were made with a 2D heteronuclear COSY spectrum (Table 1), and the diagnostic glycosylation effects [18,19] for the residues were calculated. The glycosylation effects in the ^{13}C NMR spectrum were also in agreement with the NOE: large in a module negative effect for C-4 (−3.7 ppm) of residue **C** and a small positive effect for C-4 (+0.5 ppm) of residue **A**. The

Table 2
NOE data [a] for the *E. coli* O128 polysaccharide

Residue		Proton	Pre-irradiated proton					
			A, H-1	**B**, H-1	**C**, H-1/**D**, H-1	**C** *, H-1	**D** *, H-1	**E**, H-1
→ 3)-β-D-Gal*p*NAc-(1 →	**(A)**	H-2	+					
		H-3	+		+		+	
		H-5	+					
→ 4)-α-D-Gal*p*-(1 →	**(B)**	H-2		+				
		H-4	+					
→ 3)-β-D-Gal*p*NAc-(1 →	**(C)**	H-2			+	(+) [b]		
		H-3		+	+	+		
		H-4		+				
		H-5			+	+		
→ 6)-β-D-Gal*p*-(1 →	**(D)**	H-2			+		(+) [b]	+
		H-3			+		+	
		H-5			+		+	
		H-6a, 6b			+	+		
α-L-Fuc*p*-(1 →	**(E)**	H-2						+

[a] The test was performed using standard Bruker Software NOEMULT. * Pre-irradiation of one of the components of partially overlapped anomeric proton signals of residues **C** and **D**.
[b] Pseudo-INDOOR signal.

different absolute configurations of the α-fucopyranosyl group (**E**) and the galactopyranosyl residue (**D**) bearing this group may be established from the positive glycosylation effect for C-3 of residue **D** (+0.8 ppm).

The results of compositional and methylation analyses together with the predominant set of signals in the NMR spectra suggested that the structure of the O128 polysaccharide contains branched pentasaccharide repeating units having the following structure:

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

A **B** **C** 2 ↑ **D** 1 α-L-Fuc*p* **E**

3. Experimental

Bacterial strain and cultivation.—The *E. coli* O128 strain used in this study was obtained from the culture collection of the Max-Planck-Institut für Immunbiologie (Freiburg, Germany). The bacteria were grown in a laboratory fermentor in a tryptose–tryptone medium (pH 6.8) containing yeast extract and D-glucose as carbon source at 37°C under aerobic conditions, and harvested at the beginning of the stationary phase.

Isolation of the lipopolysaccharide and preparation of O-specific polysaccharide.—LPS was extracted from the lyophilized cells by the hot phenol–water extraction procedure [6]. The crude LPS was subjected to ultracentrifugation and, from the

supernatant solution, the O128 LPS was obtained by fractional precipitation with Cetavlon [6,7]. The O-specific polysaccharide was prepared by mild acid hydrolysis of LPS with aq 2% AcOH for 2 h at 100°C followed by gel-permeation chromatography on a Sephadex G-50 column as described previously [9].

Analytical methods.—Neutral sugars were liberated by hydrolysis with 2 M CF_3CO_2H at 120°C for 2 h. The products were then converted into their alditol acetates and analysed by GLC on a Hewlett–Packard model 5730 A gas chromatograph equipped with a flame-ionisation detector and a model HP-3380 A electronic integrator. Peaks were identified by cochromatography with authentic standards. The alditol acetates were resolved on a glass column (1.8 × 6 mm) containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 170°C. Amino sugars liberated by hydrolysis, using 4 M HCl at 100°C for 6 h, were determined by a Waters Associates HPLC system on a PICO TAG C_{18} reversed-phase column after preparation of their phenylthiocarbamoyl derivatives. Gas–liquid chromatography–mass spectrometric (GLC-MS) analysis was performed on a Hewlett–Packard 5988 A automatic GLC-MS system, using a fused-silica SP-2330 capillary column (25 m × 0.25 mm i.d.) and a temperature programme starting at 50°C followed by an increase of 70°C/min to 150°C then 5°C/min to 240°C: ion energy, 70 eV; ion-source temperature, 200°C; injector port temperature, 300°C. Paper chromatography was performed on Whatman No. 1 and No. 3MM papers. Solvent systems used were **1**, 8:2:1 EtOAc–pyridine–water; **2**, 6:4:3 1-butanol–AcOH–water (upper layer); and **3**, 5:5:1:3 EtOAc–pyridine–AcOH–water. Thin layer chromatography was done with glass plates coated with Silica Gel G (Merck), using 8:1 $CHCl_3$–MeOH and 3:2 MeCN–water as the mobile phases.

Gel filtrations were performed on columns of Sephadex G-50 (2 × 70 cm), using a Gilson fraction collector, model 201, and the eluate was monitored by a Waters Associates differential refractometer (model 403) fitted with a recorder. The gel filtration properties of the eluates are expressed in terms of their distribution coefficient K_{av}; $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_e is the elution volume of the specific material, V_o is the void volume of the system, and V_t is the total volume of the system.

Methylation analysis.—Samples (2–4 mg) were methylated according to Ciucanu and Kerek [8], and the products were isolated by partition between CH_2Cl_2 and water. The products from the organic phase were further purified by passing through a Sep-Pak C_{18} cartridge [20]. Methylated products were hydrolysed with 2 M CF_3CO_2H at 120°C for 2 h, reduced with $NaBD_4$, acetylated with Ac_2O in pyridine, and analysed by GLC or GLC-MS or both.

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were recorded with a Bruker WM-300 spectrometer in D_2O at 70°C, using acetone (δ_H 2.225; δ_C 31.45) as the internal standard. Standard Bruker software was used for homonuclear H,H shift-correlated spectroscopy (COSY), as well as for one- and two-step H-relayed homonuclear two-dimensional H,H-COSY. The nuclear Overhauser effect spectra were recorded in the truncated driven (TOE) mode [21] with the Bruker NOEMULT programme. The relaxation delay (D1) was 1 s, the irradiation time of every component of the multiplets (D2) was 0.1 s, and the total preirradiation time for whole multiplets was 1.0–1.2 s.

Acknowledgements

We thank Professor K. Jann and Dr B. Jann (Max-Planck-Institut für Immunbiologie, Freiburg) for providing the bacterial strain and for their active interest in this work. We also thank Professor N. Roy, Professor B.P. Chatterjee, and Dr A.K. Guha of the Department of Biological Chemistry, I.A.C.S., Calcutta for their constant help and encouragement during the work, and Dr O. Holst (Forschunginstitut, Borstel) and Mr D. Borowiak (Max-Planck-Institut für Immunbiologie, Freiburg) for GLC-MS and amino sugar analyses. This work was partially supported by grant No. 93-03-5839 of the Russian Foundation in Fundamental Sciences.

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