Carbohydrate Research, 254 (1994) 221–228
Elsevier Science B.V., Amsterdam

Structure of the O-specific side chain of the lipopolysaccharide from *Escherichia coli* O126

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(Received April 6th, 1993; accepted August 16th, 1993)

ABSTRACT

The polysaccharide isolated from *Escherichia coli* O126 lipopolysaccharide contains D-galactose, D-mannose, L-fucose, and 2-acetamido-2-deoxy-D-glucose in the molar ratios 1:1:1:1. The structure of the O-antigen of the lipopolysaccharide from *E. coli* O126 was established by compositional analysis, partial degradation, methylation analysis, and nuclear magnetic resonance spectroscopy. These studies revealed that the O-antigen is branched and built up of tetrasaccharide repeating units having the following structure:

$$\rightarrow 2)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \beta\text{-L-Fuc}p \end{array}$$

INTRODUCTION

Escherichia coli is a complex group of bacteria and many of its serotypes are important human pathogens causing extraintestinal and intestinal infections[1–3]. The serological classification of the *E. coli* group is mainly 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[4]. So far, 171 different O-serotypes of *E. coli* are known[5], 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*[6]. The enteropathogenic serotypes are known to be associated with infantile diarrhoea[3]. This communication contains the results of chemical investigations

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SSDI 0008-6215(93)E0326-V

carried out on the O-specific polysaccharide isolated from an enteropathogenic strain of *E. coli* O126.

EXPERIMENTAL

Bacterial strain.—*E. coli* O126 (strain 21750) used in this study was obtained from the collection of the Max-Planck Institut für Immunobiologie (Freiburg). The bacteria were grown in a laboratory fermenter in a tryptose–tryptone medium (pH 6.8) containing yeast extract and D-glucose as carbon source, at 37°C under aerobic conditions, and were harvested at the beginning of the stationary phase.

Isolation and purification of lipopolysaccharides.—Lipopolysaccharide was extracted from the acetone-dried bacterial mass by the Westphal procedure[7]. The crude lipopolysaccharide was purified by repeated ultracentrifugation at 105 000 *g*. The final pellet was dissolved in water, the solution was lyophilised, and the material was termed LPS(I).

From the supernatant solution of the first ultracentrifugation, an additional lipopolysaccharide [LPS(II)] was obtained by fractional precipitation with Cetavlon[8]. The lipid part of the lipopolysaccharide was cleaved by mild hydrolysis (aq 2% acetic acid, 100°C, 2 h) and the O-specific polysaccharide was isolated by gel chromatography on Sephadex G-50 as described previously[9].

Analytical methods.—Quantitative colorimetric methods used were (*a*) the phenol–H_2SO_4 method for neutral glycoses[10], (*b*) the modified Elson–Morgan method for aminodeoxyhexoses[11], and (*c*) the cysteine–H_2SO_4 method for 6-deoxyhexoses[12]. Spectrophotometric measurements were made with a Hitachi model 100-60 spectrophotometer. GLC was performed with a Hewlett–Packard 5730A gas chromatograph fitted with a flame-ionisation detector and a model HP-3380A electronic integrator. Glass columns (1.8 m × 6 mm) packed with *A*, 3% ECNSS-M on Gas Chrom Q (100–120 mesh); *B*, 3% OV-225 on Gas Chrom Q (100–120 mesh); and *C*, 3% Poly-A 103 on Gas Chrom Q (100–120 mesh) were used for GLC. Gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was performed on a Hewlett–Packard 5988A 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*, 4:1:5 1-butanol–acetic acid–water (upper layer); and *3*, 5:5:1:3 EtOAc–pyridine–acetic acid–water. Thin layer chromatography (TLC) was performed on 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) or Bio-Gel P-2 (200–400 mesh) (1.6 × 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.

Optical rotations were measured with a Perkin–Elmer 241 MC spectropolarimeter and concentrations were made by rotary evaporation below 45°C.

^{13}C NMR and ^{1}H NMR spectroscopy was performed with a Bruker AMX-500 spectrometer in the Fourier-transform mode with and without proton decoupling. Spectra were run at 50°C with sodium 3-trimethylsilyl(2,2,3,3-$^{2}H_4$)propionate as external standard.

Hydrolysis methods.—Total hydrolysis of oligo- and poly-saccharide samples was carried out in sealed tubes with 2 M CF_3CO_2H at 120°C for 2 h, followed by evaporation to dryness by flushing with N_2. Neutral sugars were determined as their alditol acetates by GLC. Amino sugars liberated by hydrolysis, using 4 M HCl at 100°C for 6 h, were estimated on an amino acid analyzer (Alpha plus 4151; LKB Products, Bromma, Sweden) or by a Waters Associates HPLC system on a PICO TAG C_{18} reverse-phase column after preparation of their phenylthiocarbamoyl derivatives[13].

Selective hydrolysis (removal of L-fucopyranosyl nonreducing end groups) of the O-polysaccharide was done with 0.5 M CF_3CO_2H at 100°C for 1 h, followed by dialysis and lyophilisation.

Partial hydrolysis of the O-polysaccharide was made with 0.5 M HCl at 100°C for 35 min. After removal of the acid, the hydrolysate was chromatographed on Whatman No 3MM papers (solvent system *2*) when spots corresponding to monosaccharides and poorly resolved slow-moving spots of oligosaccharides were obtained. The zone containing the oligosaccharide fractions was cut out and eluted with ethanolic water, and then freeze-dried. The oligosaccharide mixture was then fractionated on a column of Bio-Gel P-2 when two oligosaccharides (**4** and **5**) were obtained as major fractions. Each oligosaccharide was further purified by passing through the same column of Bio-Gel P-2 which was eluted with water, and the appropriate fractions were freeze-dried. Both the oligosaccharides were found to be chromatographically homogeneous (PC).

Methylation analysis.—Samples (2–4 mg) were methylated according to Ciucanu and Kerek[14], and the products were isolated by partition between CH_2Cl_2 and water. The products were further purified by passing through a Sep-Pak C_{18} cartridge[15]. 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.

Periodate oxidation.—Polysaccharide (40 mg) in water (7 mL) containing sodium metaperiodate (100 mg) was kept for 3 days in the dark at 24°C. The excess of periodate was destroyed with ethylene glycol (0.15 mL) and the resulting polyaldehyde was reduced with $NaBH_4$ (200 mg) at 24°C for 16 h. Excess of borohydride was destroyed by acidification with aq 50% acetic acid and the product was recovered by lyophilisation of the dialysed mixture. The polyol was treated with 0.5 M CF_3CO_2H (10 mL) at 24°C for 24 h. The hydrolysate was evaporated at 24°C,

the residue was repeatedly evaporated with water and then reduced with $NaBH_4$ (24°C, 4 h), the solution was acidified with acetic acid to pH 4.5, and the oligosaccharide **2** was isolated by gel filtration chromatography on Bio Gel P-2.

Enzymatic hydrolysis.—Enzymatic hydrolysis of oligosaccharide **2** (10 mg) was performed in 0.1 M sodium phosphate buffer (pH 7.2, 5 mL) separately with α-D-galactopyranosidase (EC 3.2.1.22; *E. coli*, Sigma) and β-D-galactopyranosidase (EC 3.2.1.23; *E. coli*, Sigma) at 37°C for 48 h. The enzyme was deactivated by heating on a water bath for 5 min and the mixture was then filtered. The filtrate was freeze-dried and analysed by GC of alditol acetates.

N-*Deacylation and deamination of the polysaccharide.*—Polysaccharide (30 mg) was dissolved in 1 M NaOH (3 mL) containing $NaBH_4$ (3 mg). The mixture was heated in a sealed tube at 100°C for 2 h. The excess of NaOH was neutralised with cold 2 M HCl and the *N*-deacylated product (20 mg) was isolated by lyophilisation of the dialysed mixture. Aqueous 5% sodium nitrite (2 mL) and aq 33% acetic acid (2 mL) were added to the solution of the *N*-deacylated product (12 mg) in water (1.5 mL). The mixture was stirred for 2 h at room temperature, diluted with water (3 mL), and freeze-dried. The residue was treated with $NaBH_4$ (60 mg) in water (2 mL) at room temperature overnight. Excess of reagent was decomposed with Dowex-50 (H^+) resin and the solution evaporated. The residue, after removal of boric acid as the methyl ester, was purified by chromatography on Bio Gel P-2 followed by lyophilisation.

RESULTS AND DISCUSSION

Preparation of LPS and its degraded products.—Extraction of *E. coli* serotype O126 cells (10 g dry wt) by the hot phenol–water extraction procedure, followed by repeated ultracentrifugation, afforded an LPS preparation (I) as a pellet (130 mg). From the supernatant solution of the ultracentrifugation, a second LPS preparation (II) (43 mg) was obtained by fractional precipitation with Cetavlon.

The chemical composition of the polysaccharide part of both LPS(I) and LPS(II) were found to be the same. These results were also observed in the case of *E. coli* O124[16] and O58[17]. Isolation and characterisation of the O-specific polysaccharide was carried out with LPS(I) preparation. Partial hydrolysis of LPS(I) (100 mg) with hot dilute acetic acid yielded an insoluble lipid A (20 mg), and gel permeation chromatography of the water-soluble products on Sephadex G-50 gave an O-polysaccharide (K_{av} 0.04, 38 mg), a core oligosaccharide (K_{av} 0.65, 10 mg), and a fraction containing phosphate and 3-deoxy-D-*manno*-octulosonic acid (K_{av} 0.90, 5 mg).

Structural studies of the O-specific polysaccharide.—The lyophilised O-polysaccharide had $[\alpha]_D$ +28.1° (*c* 1.1, H_2O). Anal. Found: C, 41.18; H, 6.01; N, 2.14. Paper chromatography of the polysaccharide hydrolysate as well as GLC analysis of the alditol acetates showed that the polysaccharide from *E. coli* O126 consists of D-galactose, D-mannose, L-fucose, and 2-acetamido-2-deoxy-D-glucose. The

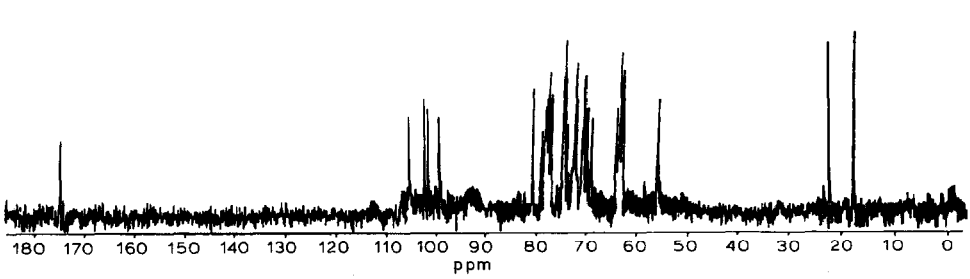


Fig. 1. The ^{13}C NMR spectrum of the O-specific polysaccharide of *E. coli* O126.

quantitative determinations of the constituent sugars showed that they are present in equimolar ratios. The specific optical rotation values of the sugar monomers, isolated from the polysaccharide hydrolysate by preparative paper chromatography, enabled assignment of the D configuration to the galactose, mannose, and 2-acetamido-2-deoxyglucose, but L to the fucose residues.

The ^{13}C NMR spectrum (Fig. 1) of the O-specific polysaccharide contained signals for four anomeric carbons at 99.9, 102.3, 102.8, and 105.4 ppm, having $^1J_{CH}$ values of 173, 162, 160, and 163 Hz, respectively, indicating that one glycosidic linkage in the repeating unit of the O-polysaccharide has the α configuration and the other three have the β configuration. The spectrum also contained signals for one methyl group of a 6-deoxy sugar (fucose) at 17.9 ppm, three hydroxymethyl groups (C-6 of galactose, mannose, and glucosamine) at 63.1–63.6 ppm, C-2 of the amino sugar at 55.8 ppm, other carbons in the region 69.3–81.3 ppm, as well as one acetamido group (CH_3 at 22.6 ppm and CO at 176.2 ppm). These data showed that the O-specific polysaccharide is built up of tetrasaccharide repeating units, consistent with the results of acid hydrolysis.

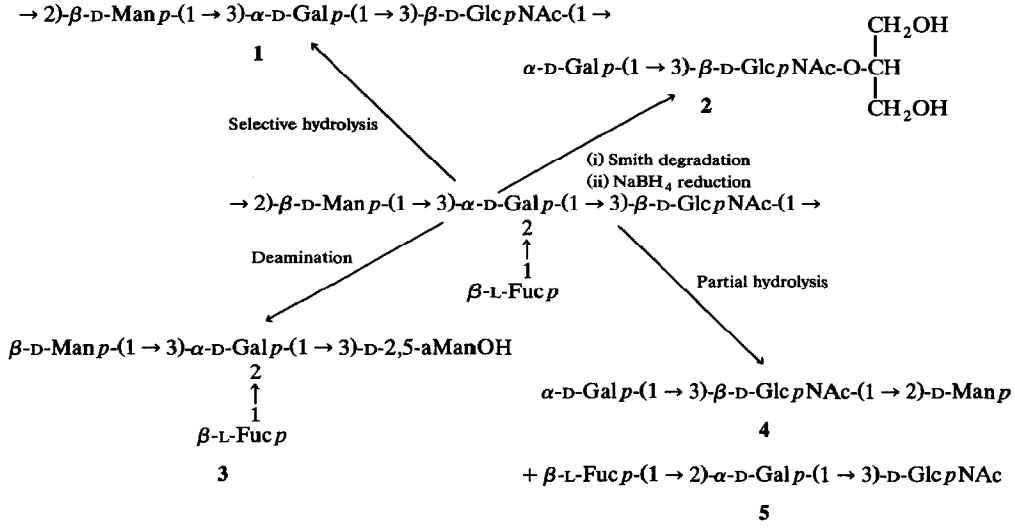
Results of methylation analysis of the O-specific polysaccharide and the derived oligosaccharides are given in Table I. Alditol acetates obtained from the hydro-

TABLE I

Methylation analysis of the O126 polysaccharide and its degraded products [a]

Methylated sugar	Molar ratio					
	I	II	III	IV	V	VI
2,3,4,6-Me_4-Gal	– [b]	–	1.0	–	1.0	–
2,3,4,6-Me_4-Man	–	–	–	1.0	–	–
3,4,6-Me_3-Man	1.0	1.0	–	–	1.0	–
2,4,6-Me_3-Gal	–	0.8	–	–	–	–
2,3,4-Me_3-Fuc	1.0	–	–	1.0	–	1.0
3,4,6-Me_3-Gal	–	–	–	–	–	0.8
4,6-Me_2-Gal	0.8	–	–	0.8	–	–
4,6-Me_2-GlcNAc	0.6	0.7	0.7	–	0.6	0.7

[a] 2,3,4,6-Me_4Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, etc. I, O126 polysaccharide; II, selective acid-hydrolysed polysaccharide; III, Smith-degraded and $NaBH_4$ reduced polysaccharide; IV, deaminated polysaccharide; V and VI, oligosaccharides **4** and **5**, respectively. [b] –, Component absent.



Scheme 1. Fragmentation products of the *E. coli* O126 polysaccharide.

lysate of the methylated polysaccharide revealed (GLC and GLC–MS) derivatives of 3,4,6-tri-*O*-methylmannose, 2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamidoglucose, 4,6-di-*O*-methylgalactose, 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 galactopyranoside residue substituted at O-2 and O-3 represents a branch point, the mannopyranose residue is substituted at O-2, while the glucosamine residue in its pyranosidic form is substituted at O-3. Selective acid hydrolysis (0.5 M CF_3CO_2H, 1 h, 100°C) followed by dialysis and lyophilisation afforded a modified polysaccharide (**1**), which was found to be composed of D-galactose, D-mannose, and 2-acetamido-2-deoxy-D-glucose in equimolar proportions. Methylation analysis of **1** gave 2,4,6-tri-*O*-methylgalactose together with the other partially methylated hexoses found in the methylation analysis of the native polysaccharide except for 4,6-di-*O*-methylgalactose and 2,3,4-tri-*O*-methylfucose. Appearance of 2,4,6-tri-*O*-methylgalactose shows that the fucopyranosyl group was the single substituent attached to the galactopyranosyl residue at its 2-position.

The sequence of the monosaccharide residues in the polysaccharide was determined by characterisation of fragmentation products (Scheme 1) of the polysaccharide from Smith degradation, deamination, and partial acid hydrolysis.

The periodate-oxidised and reduced ($NaBH_4$) O-polysaccharide on Smith-type hydrolysis, $NaBH_4$ reduction, and subsequent gel chromatographic separation of the hydrolysate on Bio-Gel P-2 gave a single product (**2**) having $[\alpha]_D$ +53° (*c* 0.83, H_2O). Compositional analysis of the product showed equimolar proportions of

D-galactose, 2-acetamido-2-deoxy-D-glucose, and glycerol. Methylation analysis of the Smith-degraded product showed the presence of a terminal galactose and a 3-linked 2-acetamido-2-deoxy-D-glucose residue (Table I). The ^{1}H NMR spectrum of **2** showed two anomeric signals at 5.3 ($J_{1,2}$ 3 Hz) and 4.7 ppm ($J_{1,2}$ 7.5 Hz), thus indicating the presence of one α-linked and one β-linked sugar residue. Enzymatic treatment of the oligosaccharide **2**, using α-D-galactopyranosidase, completely cleaved the galactosyl unit whereas similar treatment with β-D-galactopyranosidase did not, so the ^{1}H signal at 5.3 ppm was assigned to the α-D-galactopyranosyl residue and that at 4.7 ppm to the 2-acetamido-2-deoxy-β-D-glucose residue. These data indicate that the structure α-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc-(1 → 2)-glycerol represents the periodate-resistant moiety of the O126 polysaccharide.

To prepare the deaminated fragment **3**, the O-polysaccharide was *N*-deacetylated and deaminated using sodium nitrite in aqueous acetic acid, followed by gel permeation chromatography on Bio-Gel P-2. Methylation analysis of **3** showed (Table I) that 3,4,6-tri-*O*-methylmannose found in the analysis of the original polysaccharide was replaced by 2,3,4,6-tetra-*O*-methylmannose. This result indicates that the 2-acetamido-2-deoxy-D-glucose moiety is linked to O-2 of the mannopyranosyl residue.

Partial hydrolysis of the O126 polysaccharide in 0.5 M HCl for 35 min at 100°C yielded two major oligosaccharides (**4** and **5**) which were separated by gel chromatography on Bio-Gel P-2. Composition and methylation analysis of **4** and **5** (Table I) together with the result of Smith-degradation studies of the O126 polysaccharide indicated that D-Gal*p*-(1 → 3)-D-Glc*p*NAc-(1 → 2)-D-Man and L-Fuc*p*-(1 → 2)-D-Gal*p*-(1 → 3)-D-Glc*p*NAc are the respective structures of oligosaccharides **4** and **5**. All these results led to the branched tetrasaccharide structure of the O126 polysaccharide.

Based on reported[20,21] ^{13}C NMR chemical shift values as well as the known correlations between glycosylation effects, the absolute configuration of the sugar components, and the configuration of the glycosidic linkage[22], tentative assignments of ^{13}C NMR chemical shift values were made for α-D-galactose (99.9 ppm), 2-acetamido-2-deoxy-β-D-glucose (102.9 ppm), β-D-mannose (102.3 ppm), and β-L-fucose (105.3 ppm), respectively.

Calculation of optical rotation.—The specific optical rotation of the O126 polysaccharide was calculated by Klyne's rule[18,19], and the calculated value {$[\alpha]_D$ +24°} was found to be in good agreement with the observed value {$[\alpha]_D$ +28.1°}, thus supporting the above assignments of absolute configurations of the constituent monosaccharides.

ACKNOWLEDGMENTS

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 our Department for their constant help and encouragement during the work, and Dr. O. Holst (Forschungsinstitut, Borstel), Mr. D. Borowiak and Miss V. Person (Max-Planck Institut für Immunbiologie, Freiburg) for GLC–MS and amino sugar analyses. NMR spectral studies were carried out with assistance from the Tata Institute of Fundamental Research, Bombay.

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