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The characterization of the O-antigen of *Escherichia coli* 064 : K99 lipopolysaccharide

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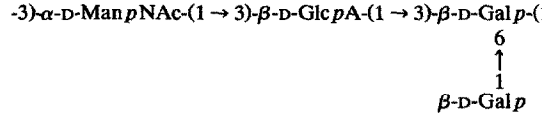
ABSTRACT

The structure of the O-polysaccharide of the smooth lipopolysaccharide (LPS) produced by *Escherichia coli* O64 : K99 was investigated by SDS–PAGE, composition, periodate oxidation, methylation, partial hydrolysis, and 1D and 2D nuclear magnetic resonance analyses, made on the native o-chain and its reduction and periodate degradation products.

The *E. coli* O64 antigenic O-chain was found to be a high molecular weight glycan composed of D-galactose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-mannose (2 : 1 : 1 : 1) and was a polymer of branched pentasaccharide repeating units having the structure:



INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* O64 have been associated with enteric colibacillosis in pigs[1]. The determination of the structure of the lipopolysaccharide (LPS) was undertaken to provide information on the structural basis for the serological antigenic O : 64 specificity which resides in the polysaccharide moiety of its cellular LPS.

RESULTS AND DISCUSSION

Extraction of fermenter-grown cells of *E coli* O64 : K99 (NRCC 4091) by a modified[2] aqueous phenol method, followed by ultracentrifugation of the concen-

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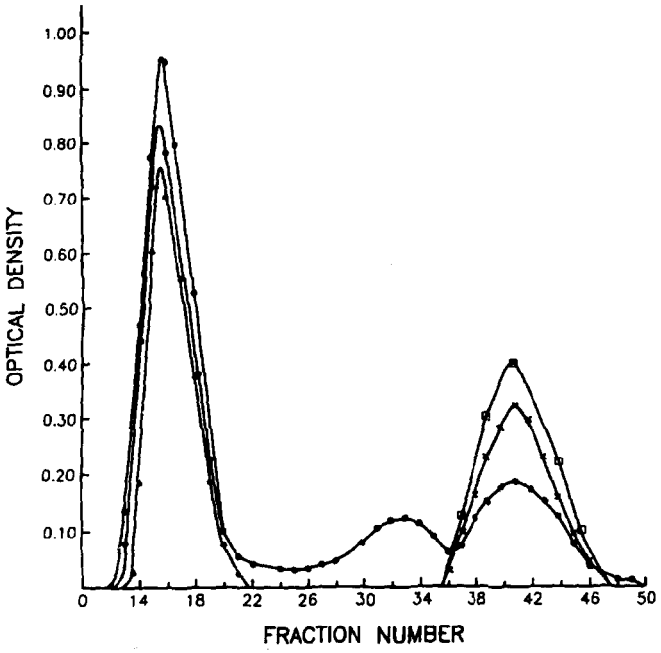


Fig. 1. Sephadex G-50 chromatographic separation of the water-soluble products from the mild hydolysis of *E. coli* O64;K99 lipopolysaccharide preparation. Neutral glycose[11] (●-●-●); 2-amino-2-deoxyhexose[12] (-○-○-○-); 3-deoxy-2-octulosonate[13] (-×-×-×-), hexuronic acid[14] (-▲-▲-▲-), and phosphate[16] (-□-□-□-)

trated, dialyzed separated phenol and water layers, afforded precipitated gels of LPS in 0.7 and 8% yields (based on dry cell weight) from the respective phases.

SDS–PAGE analysis of the phenol phase LPS indicated it to contain essentially lipooligosaccharide (R-LPS), and it was not studied further. The aqueous-phase LPS was indicated to be S-type LPS and gave a ladder-type banding pattern with a band spacing typical of an LPS containing a repeating pentasaccharide repeating unit[3].

Hydrolysis of the aqueous-phase LPS with hot 2% acetic acid (100°C, 2 h) gave an insoluble lipid A (38%), and Sephadex G-50 gel-filtration chromatgraphy of the water-soluble products (Fig. 1) yielded an O-polysaccharide (O-PS) (27%, K_{av} 0.03), a core oligosaccharide (4%, K_{av} 0.42), and a fraction (13%, K_{av} 0.97) containing 3-deoxy-D-*manno*-2-octulosonic acid.

The O-PS had $[\alpha]_D$ +14.5° (*c* 1.41, H_2O), and elemental analysis gave Anal. Found: C, 39.93; H, 5.34; N; 2.34%; and ash, nil. Quantitative analysis of the acid-hydrolysis products of the O-PS was made by GLC–MS methods (Table I), with characterization of configurations made by capillary GLC of their trimethylsilylated derivatives of the glycoses and their 2-(*S*)-butyl glycosides[4]. The configuration of the aminoglycoses was further confirmed by their ninhydrin degradation to yield only D-arabinose[5]. The characterization for the D-glucuronic acid component

TABLE I

Quantitative analysis of the *E. coli* O:64 LPS O-polysaccharide (O-PS), carboxyl-reduced ($NaBD_4$) O-PS (R-O-PS), and periodate-oxidized R-O-PS (PR-O-PS) by the GLC–MS alditol acetate derivative method [a]

Acetylated alditol derivative	t_{GA}	O-PS	R-O-PS	PR-O-PS
1,2,3,4,5,6-Hexa-*O*-acetyl-galactitol	1.00	1.00	1.00	1.00
1,2,3,4,5,6-Hexa-*O*-acetyl-D-glucitol-6-d_2	1.06		0.51	0.95
2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol	1.32	0.47	0.45	0.92
2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-mannitol	1.42	0.45	0.45	0.90

[a] Retentions times are quoted relative to 1,2,3,4,5,6-hexa-*O*-acetyl-galactitol ($t_{GA} = 1.00$). See ref 17 for details of the GLC–MS alditol acetate method.

was confirmed through the identification of D-glucose-6-d_2 in the hydrolysate of the carboxyl-reduced ($NaBD_4$) O-PS. The O-PS was thus composed of D-galactose (D-Gal), 2-amino-2-deoxy-D-mannose (D-ManN), 2-amino-2-deoxy-D-glucose (D-GlcN), and D-glucuronic acid (D-GlcA) in the molar ratios 2:1:1:1. Quantitative analysis of the carboxyl-reduced ($NaBD_4$) O-PS (Table I) {$[\alpha]_D$ +21° (c 1.01, H_2O)} confirmed the determined composition and suggested that the O-PS was composed of repeating pentasaccharide units.

Methylation analysis of the O-PS and the carboxyl-reduced ($NaBD_4$) O-PS (Table II) indicated that it contained the structural residues D-Gal*p*-(1→, →3)-D-Glc*p*NAc-(1→, →3)-D-Man*p*NAc-(1→, →3)-D-Glc*p*A-(1→, and →3,6)-D-Gal*p*-(1→, and that the repeating units are branched pentasaccharides containing D-Gal*p* nonreducing end-groups and 3,6-di-*O*-substituted D-Gal*p* branch residues.

Periodate oxidation of the native O-PS, followed by Smith-type hydrolysis of the reduced ($NaBH_4$) product[6] and Sephadex G-50 chromatography, gave a polymer (82%, K_{av} 0.05) having $[\alpha]_D$ +10° (*c* 0.70, H_2O), composed of D-Gal, D-GlcNAc, D-ManNAc, and D-GlcA (1:1:1:1). Methylation analysis (Table II) showed the oxidized product to be a polymer of unbranched tetrasaccharide units containing

TABLE II

Methylation analyses of E. *coli* O64 LPS O-polysaccharide (O-PS) and its carboxyl-reduced (R-O-PS) and periodate-oxidized R-O-PS (PR-O-PS) [a]

Methylated alditol acetate derivative	t_{GM}	Molar ratio		
		O-PS	R-O-PS	PR-O-PS
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-1-*d*	1.09	0.88	0.90	
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol-1,6,6-d_3	1.41		0.98	1.0
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol-1-*d*	1.54			1.0
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol-1-*d*	2.28	1.00	1.00	
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol-1-*d*	3.60	0.91	0.89	0.82
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-mannitol-1-*d*	3.66	0.86	0.81	0.76

[a] Retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-terta-*O*-methyl-D-glucitol ($t_{GM} = 1.00$)

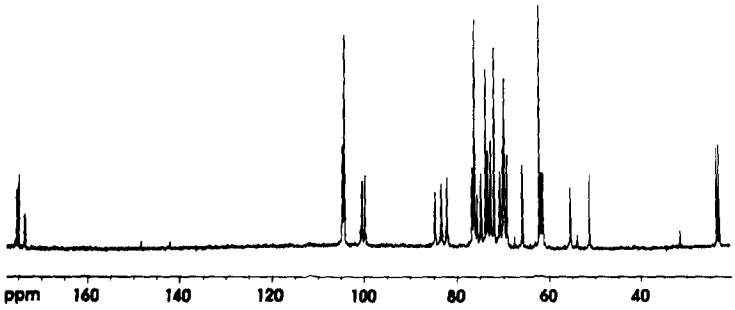


Fig. 2. ^{13}C NMR spectrum (125 MHz) at 310 K of the LPS O-polysaccharide of *E. coli* O64;K99.

only (1 → 3) glycosidic linkages thus showing that in the native O-PS, the nonreducing D-Gal*p* end-groups were present as single residues substituted at the O-6 position of the D-Gal*p* branch points. A similar periodate oxidation study made on the carboxyl-reduced ($NaBD_4$) O-PS gave a product which by composition (Table I) and methylation analysis (Table II) was, as expected, found to be a polymer of a tetrasaccharide unit of (1 → 3)-linked D-Gal*p*, D-Glc*p*NAc, D-Man*p*NAc, and D-Glc*p* (1:1:1:1) residues. This degraded PS was not further oxidized by periodate.

In agreement with the proposed pentasaccharide unit in the O-PS, its ^1H NMR spectrum showed *inter alia*, five anomeric protons signals at δ 5.231 (1 H, $J_{1,2}$ 1.4 Hz), 4.760 (1 H, $J_{1,2}$ 8.0 Hz), 4.665 (1 H, $J_{1,2}$ 8.3 Hz), 4.477 (1 H, $J_{1,2}$ 8.0 Hz), 4.449 (1 H, $J_{1,2}$ 8.0 Hz), together with characteristic signals for the methyl signals from *N*-acetyl substituents at δ 2.035 (s, 3 H) and 1.988 (s, 3 H). Consistent with the ^1H NMR spectrum, the ^{13}C NMR spectrum of the O-PS (Fig. 2) showed *inter alia*, five anomeric carbon signals at δ 104.5 ($J_{C,H}$ 170 Hz), 104.2 ($J_{C,H}$ 161 Hz), 104.0 ($J_{C,H}$ 162 Hz), 100.2 ($J_{C,H}$ 176 Hz), and 99.5 ($J_{C,H}$ 162 Hz), signals at δ 23.2 and 22.7 (CH_3CONH) from *N*-acetyl substituents associated with resonances at δ 55.0 and 50.9 arising from C-2 of component 2-acetamido-2-deoxyglycoses, and three carbonyl resonances at δ 175.2, 174.8, and 173.4 arising from two *N*-acetyl substituents (CH_3CONH) and the carboxyl group of the D-Glc*p*A residue.

The anomeric configurations and sequence of glycoses in the O-PS were established from NMR studies. COSY experiments[7,8] made on the O-PS allowed the unambiguous assignments of anomeric and ring proton signals (Table III). Each anomeric proton signals was labelled *a* to *e* in order of decreasing chemical shifts, and connectivities were traced via cross-peaks starting from each anomeric proton signals. ^1H NMR assignments were verified using two- and three-step relay COSY analyses. From the established D configurations of the component hexose residues and a consideration of the observed chemical shifts and coupling constants, *a* was identified as D-Man*p*NAc, *b* as β-D-Glc*p*A, *c* as β-D-Glc*p*NAc, and *d* and *e* as β-D-Gal*p* residues. Chemical shifts were refined (Table III) by simulating the ^1H NMR spectrum of the O-PS (Fig. 4). A heteronuclear ^{13}C–^1H

TABLE III

^{1}H NMR spectral data [a,b] (500 MHz) for *E. coli* O:64 LPS O-polysaccharide

Unit	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
(*a*) → 3)-α-D-Man*p*NAc-(1 →	5.231	4.489	4.226	3.782	4.056	3.841	3.841
	(1.4)	(4.5)	(9.5)	(10.0)	(2.0)	(12.3)	(6.0)
(*b*) → 3)-β-D-Glc*p*A-(1 →	4.760	3.529	3.697	3.801	3.990		
	(7.8)	(9.5)	(10.0)	(10.0)			
(*c*) → 3)-β-D-Glc*p*NAc-(1 →	4.665	3.857	3.804	3.579	3.515	3.931	3.773
	(8.3)	(10.0)	(9.0)	(9.5)	(2.5)	(12.3)	(5.5)
(*d*) → 3,6)-β-D-Gal*p*-(1 →	4.477	3.706	3.799	4.156	3.919	4.053	3.909
	(8.0)	(10.0)	(3.8)	(1.0)	(4.0)	(11.5)	(8.0)
(*e*) β-D-Gal*p*-(1 →	4.449	3.548	3.648	3.936	3.684	3.767	3.789
	(8.0)	(10.0)	(3.8)	(1.0)	(5.0)	(12.3)	(8.0)]

[a] Chemical shifts, reported in δ-units (ppm) downfield from Me_4Si_1 were measured in ppm (±0.005 ppm) at 343 K in D_2O using acetone as an internal reference (δ 2.225). NAc at δ 2.035 and 1.998. [b] $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, $J_{4,5}$, $J_{5,6}$, $J'_{6,6}$, and $J_{5,6}$, values in Hz (±0.5 Hz) are recorded in parentheses.

shift correlation (HETCOR) 2D NMR experiment allowed carbon chemical shift assignments to be made (Table IV).

With the knowledge of the linkage positions of the glycosyl residues established from methylation evidence, the sequential order of the O-PS component five glycose residues were determined from 1D NOE difference experiments[9] (Fig. 3). Irradiation of H-1*a* produced an NOE on H-2*a* and H-3*b* indicating that residue *a* is substituted through a glycosidic α-D-(1 → 3) link to residue *b*. Irradiation of H-1*b* resonance produced enhancements on H-2,3,5*b* and H-3*d* indicating that residue *b* is linked through a β-D-(1 → 3) link to residue *d*. Irradiation of H-1*d*

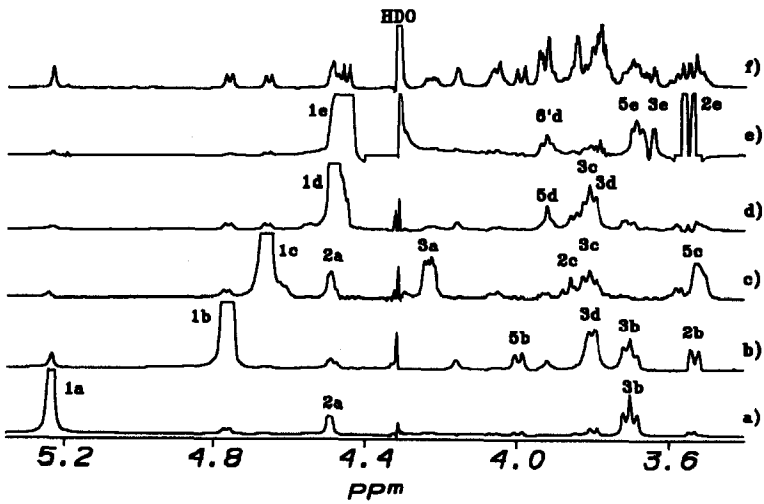


Fig. 3. NOE differences spectra for the *E. coli* O64;K99 LPS O-polysaccharide showing saturation effects: (a) on H-1*a*; (b) on H-1*b*; (c) on H-1*c*; (d) on H-1*d*; (e) on H-1*e*. Spectrum (f) is an off-resonance control spectrum measured at 343 K.

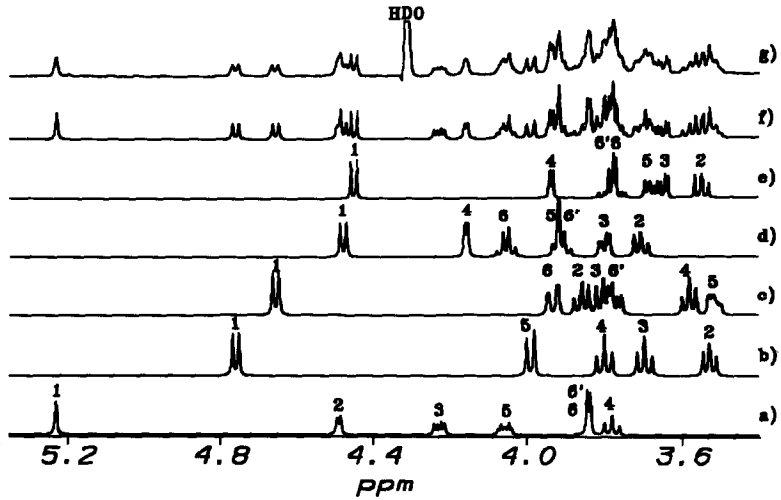


Fig. 4. ^{1}H NMR spectra (500 MHz) of the LPS O-polysaccharide of *E. coli* O64;K99 determined at 343 K. Simulated spectra for component residues are shown: (a) →3)-α-D-Man*p*NAc-(1→; (b →3)-β-D-Glc*p*A-(1→; (c) →3)-β-D-Glc*p*NAc-(1→; (d) →3,6)-β-D-Gal*p*-(1→; (e) β-D-Gal*p*-(1→. Spectrum (f) is the sum of the spectra (a, b, c, d, and e) and spectrum (g) is the observed spectrum of the LPS O-PS.

produced enhancement on its own H-3, 5*d* and across the glycosidic linkage to H-3*c* establishing that the residue *d* is β-D-(1 → 3)-linked to residue *c*. Similarily, irradiation of H-1*c* produced enhancements on its own H-2, 3, 5*c* and interresidue across ring enhancements on H-3*a* and H-2*a* indicative of residue *c* being substituted through a β-D-(1 → 3) link to residue *a*. Irradiation of H-1*e* gave enhancement of H-6′*d* (β-D-Gal*p* branch residue) and its own H-2, 3, 5*e*. Thus,

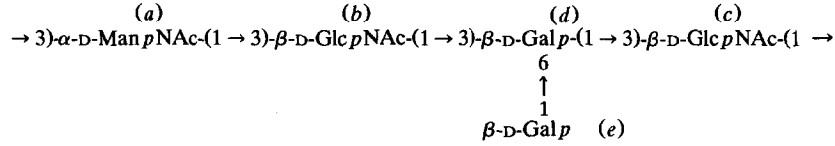
TABLE IV

^{13}C NMR spectral data [a,b] (125 MHz) for the *E. coli* O:64 LPS O-Polysaccharide

Unit	C-1	C-2	C-3	C-4	C-5	C-6
(*a*) →3)-α-D-Man*p*NAc-(1→	100.2 (176)	50.9	76.4	72.4	73.1	61.1
(*b*) →3)-β-D-Glc*p*A-(1→	104.5 (170)	72.5	81.8	65.5	75.4	
(*c*) →3)-β-D-Glc*p*NAc-(1→	99.5 (162)	55.0	84.4	69.6	76.0	61.4
(*d*) →3,6)-β-D-Gal*p*-(1→	104.2 (161)	70.5	83.0	69.0	76.0	70.0
(*e*) β-D-Gal*p*-(1→	104.0 (162)	71.7	73.4	69.5	76.0	61.8

[a] Chemical shifts, reported in δ-units (ppm) downfield from Me_4Si, were measured in ppm with reference to internal acetone (δ 31.07) in D_2O solutions at 310 K, and $J_{C,H}$ coupling constants measured in Hz are given in parentheses [b] *N*-Acetyl methyl resonances are seen at δ 23.2 and 22.7 (CH_3CONH). Carbonyl resonances at δ 175.2, 174.8 and 173.4 are due to two *N*-acetyl substituents (CH_3CONH) and one hexuronic acid carboxyl group that is not assigned.

the linear linkage sequence can be deduced as $-a-b-d-c-a-$ with the branch point residue *d* being substituted at its O-6 position by residue *e* (β-D-Gal*p*). The accumulated evidence leads to the identification of the antigenic O:64 *E. coli* LPS O-polysaccharide as a polymer of branched pentasaccharide units having the structure:



EXPERIMENTAL

Cells of *E. coli* O64:K99 (NRCC 4091; VIDO Strain no. 12) were grown in 3.7% brain–heart infusion (Difco) at 37°C in a Microfirm fermenter (New Brunswick 28 L) and were extracted by a modified enzyme–aq phenol method[5]. Subsequent isolation procedures involving ultracentrifugation (105 000*g*, 4°C, 10 h) to precipitate LPS were the same as previously described[10]. Fission of the aqueous-phase LPS (1%) in 2% acetic acid (100°C, 2 h) released insoluble lipid A, which was removed by centrifugation. The lyophilized water-soluble product was fractionated by Sephadex G-50 chromatography, and the eluate was monitored for aldose[11], aminodeoxyaldose[12], 3-deoxy-2-octulosonic acid[13], hexuronic acid[14], and phosphate[15], under previously reported conditions[10].

Glycan hydrolyses, aldose identifications, periodate oxidations, methylation analyses, and ^1H and ^{13}C NMR spectroscopy were also performed under the same conditions as previously described[10].

GLC–MS analyses were made using a Hewlett–Packard 5985 GLC–MS system using an ionization potential of 70 eV. The following conditions were employed: (A) for alditiol acetate derivatives, glass column (2 mm × 180 cm) packed with 3% (w/w) SP2340 on 80–100 mesh Supelcoport using a temperature program 180°C (delay 2 min) to 240°C at 4°C/min; (B) for methylated alditol derivatives, capillary column (0.42 mm × 25 m) 007 series bonded phase fused silica OV-17 (Quadrex) using a temperature program 200°C (delay 2 min) to 240°C at 1°C/min.

Reduction of the hexuronic acid residues in the O-PS (100 mg) was effected by three applications of the carbodiimide–$NaBD_4$ method[16] to yield a neutral P (51 mg), purified by Sephadex G-50 gel-filtration chromatography.

Optical rotations were determined using a Perkin–Elmer 141 polarimeter at 22°C using 10-cm microtubes.

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