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Structural elucidation of the O-antigenic polysaccharide from enterohemorrhagic (EHEC) *Escherichia coli* O48:H21

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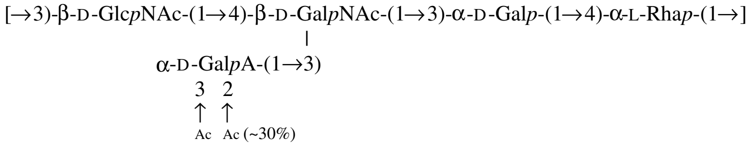
Abstract—The structure of the antigenic O-polysaccharide (O-PS) of the lipopolysaccharide (LPS) produced by the enterohemorrhagic strain of *Escherichia coli* O48:H21 (EHEC) has been elucidated. The O-PS obtained by mild acid hydrolysis of the LPS had $[\alpha]_D$ +95 (water) and was composed of L-rhamnose (L-Rha), D-galactose (D-Gal), 2-amino-2-deoxy-D-glucose (D-GlcN), 2-amino-2-deoxy-D-galactose (D-GalN), and D-galacturonic acid (D-GalA) (1:1:1:1:1). From the results of methylation analysis, mass spectrometry, 2D NMR, and DOC-PAGE, the O-PS was shown to be a high molecular mass polymer of a repeating pentasaccharide unit having the structure:



$$\begin{array}{l}[\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]\\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |\\ \qquad\qquad\quad \alpha\text{-D-Gal}p\text{A-}(1\rightarrow 3)\\ \qquad\qquad\qquad\; 3 \;\; 2\\ \qquad\qquad\qquad\; \uparrow \;\; \uparrow\\ \qquad\qquad\qquad\; \text{Ac} \;\; \text{Ac} \; (\sim 30\%)\end{array}$$

The D-Gal*p*A non-reducing end groups in the O-PS were partially O-acetylated (~30%) at the O-2 and O-3 positions and the degree of acetylation was variable from batch to batch cell production.

Keywords: *Escherichia coli* O48; Polysaccharide; LPS O-antigen

1. Introduction

Escherichia coli is best known for its ability to cause intestinal diseases[1] and six classes of the bacterium that cause diarrheal diseases are recognized as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC), with each group manifesting distinct pathogenesis, and with each class falling within serological lipopolysaccharide (LPS) specific antigenic O-polysaccharide (O-PS) based groups. *E. coli* are serologically classified by a modified Kauffman scheme based on their O (somatic), H (flagellar), and K (capsular) surface profiles[2] and serological characterization of clinical isolates of *E. coli* by a combination of O and H antigens is found to be associated reproducibly with clinical sequel, although the antigens themselves are not a significant virulence factor.

E. coli serotype O157:H7 emerged in the early 1980s, and is now the best known EHEC pathogen.[3] It is the frequent cause of severe or fatal intestinal disease and is associated with hemolytic–uremic syndrome (HUS), a multisystemic disorder that is characterized by the onset of acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia. The majority of outbreaks of human *E. coli* O157:H7 HUS cases resulted from the consumption of undercooked meat, raw milk, water, other contaminated food, by direct contact with animals or people infected with the bacterium and EHEC epidemiology is invariably associated with the *E. coli* being an

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intestinal reservoir in cattle and other animals. The key virulence factors of the *E. coli* O157:H7 pathogen include verotoxins (Vt) together with effectors and adhesions associated with type III secretion systems, while the role of LPS in the EHEC pathogenesis appears to be relatively minimal. In other established cases of EHEC disease, the identification of serotypes characteristic of the diarrheagenic pathology has been recorded for *E. coli* strains displaying the following O and associated H antigens:[1] O26:H11,H32, NM; O55:H7; O103:H2; O111ab:H8, NM; O113:H21; O117:H14; and O48:H21. As an aid to diagnostic serological identification, the structure of the O-antigen is important for a molecular level understanding of its antigenic specificity, and for the rational production of diagnostic polyclonal or monoclonal antibody[4,5] and the possible development of animal vaccines.[6–8] Herein we describe the structural analysis of the O-antigen of *E. coli* O48:H21, an EHEC strain isolated from a child patient in South Australia.[9]

2. Experimental

2.1. Bacterial growth and LPS isolation

E. coli O48:H21 (NRCC 6366) was grown in 3.7% brain–heart infusion (Difco) for 18 h at 37 °C in a 24 L fermenter with 20% DO saturation rate to a late exponential phase. Following cell killing with phenol (1% final concentration) at 4 °C for 4 h, the cell mass (330 g, wet wt.) was collected by centrifugation. The cells (320 g wet weight) were extracted by vigorous stirring with 50% (w/v) aqueous phenol (700 mL) for 15 min at 65 °C and the separated water and phenol phases from the cooled (4 °C) mixture were collected by aspiration and dialyzed against running tap water until free from phenol. The lyophilized product from the aqueous phase retentate was dissolved in 0.02 M sodium acetate (120 mL, pH 7.0) and treated sequentially with RNase, DNase, and proteinase K (37 °C, 3 h each). Following removal of trace solids, the clear solution was subjected to ultracentrifugation (105,000*g*, 4 °C, 12 h) and the precipitated gel of LPS was dissolved in distilled water and lyophilized (5.20 g).

2.2. O-Polysaccharide preparation

Aqueous phase LPS (1 g) was hydrolyzed with 2% (v/v) acetic acid (100 mL, 100 °C, 2 h) and following removal of precipitated lipid A (220 mg), the lyophilized water-soluble product (680 mg) was fractionated by Sephadex G-50 chromatography and the eluant was monitored by refractive index measurement, and by colorimetric analysis for neutral aldose,[10] hexuronic acid,[11] and 2-amino-2-deoxyhexose.[12] Appropriate fractions were collected and lyophilized to yield: O-PS (K_{av} 0.04, 351 mg), core oligosaccharide (K_{av} 0.34) and a low molecular mass fraction (K_{av} 0.93) containing Kdo.

O-Deacetylation was made by treatment of the isolated O-PS (200 mg) with 10% aqueous ammonia at 37 °C for 12 h followed by dialysis and lyophilization to yield 150 mg product.

2.3. DOC-PAGE analysis

Electrophoretic analysis of LPS was made using 13% polyacrylamide gels containing 0.9% sodium deoxycholate, and the bands were detected by periodate oxidation–silver nitrate staining.[13]

2.4. Component analysis

O-PS (20 mg) was hydrolyzed with 2 M trifluoroacetic acid at 105 °C for 2 h and was concentrated to dryness in a stream of dry nitrogen. A portion of the residue (14 mg) was reduced ($NaBD_4$) and acetylated (Ac_2O, 110 °C, 2 h) and analyzed by GC–MS. The remaining residue was fractionated by preparative paper chromatography and the configurations of the individual aldose and 2-amino-2-deoxyhexose sugars were determined by GLC analysis of their acetylated (+)-2-butyl glycosides as previously described.[14] An identical component analysis was also made on the carbodiimide reduced ($NaBD_4$) O-PS in which the uronic acid component (D-GalA) was revealed as D-galactose-6d_2.

2.5. Methylation analysis

Samples (2–4 mg) dissolved in Me_2SO (1 mL) were methylated by the Ciucanu–Kerek procedure[15] and were hydrolyzed with 3 M TFA (100 °C, 1 h) or 2 M HCl (100 °C, 3 h), reduced ($NaBD_4$), acetylated, and analyzed using a Varian Saturn 200 ion-trap GC–MS instrument.

Gas chromatography was done using an Agilent 6850 chromatograph fitted with a hydrogen flame detector and a capillary DB-17 fused silica column (0.25 mm × 30 m). Temperature programs used were (A) 180 °C (delay 2 min) to 240 °C at 2 °C/min for acetylated alditols, and (B) 200 °C (delay 2 min) to 240 °C at 1 °C/min to 240 °C for acetylated methylated alditol derivatives. Retention times are recorded relative to hexa-*O*-acetyl-D-glucitol ($T_G = 1.0$) or to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol ($T_{GM} = 1.0$). The GLC mobilities and MS spectra of all prepared derivatives were identical with those of authentic reference samples.

2.6. Chromatography

Sephadex G-50 gel-filtration column (3 × 95 cm) chromatography was made using 0.05 M pyridinium acetate (pH 4.7) buffer or on Bio-Gel P-2 (1 × 35 cm) using water as the eluant. Eluates were monitored using a Waters 402 refractometer.

Preparative paper chromatographic separations were made on Whatman No. 1 filter paper using (A) 10:3:3 BuOH–EtOH–water for neutral glycoses and (B) 4:1:5 BuOH–EtOH–water for aminodeoxyaldoses. Detections were made using periodate–alkaline silver nitrate, *p*-anisidine HCl, and ninhydrin spray reagents.

2.7. NMR spectroscopy and MS spectrometry

Standard 2D acquisition parameters were used for COSY, TOCSY (mix = 80 ms), NOESY (mix = 200 ms), HSQC, and gHMBC (optimized for 5 Hz) using a Varian Innova spectrometer (400 MHz), 1800 Hz sweep width and a temperature of 25 °C. Samples were exchanged twice with D_2O and then run in 99.8% D_2O. Chemical shifts are referenced to internal acetone methyl resonances ^{1}H, 2.225 and ^{13}C, 31.07 ppm.

ESIMS was done on a Prince CE system (Prince Technologies, Netherlands) coupled to an API mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) with a microspray interface.

2.8. Periodate oxidation

Smith-type oxidation[16] of the reduced O-PS (50 mg) was done as previously described.[17]

2.9. O-PS reduction by the carbodiimide method[18]

A stirred solution of O-deacetylated O-PS (90 mg) in water (30 mL) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (300 mg) and maintained for 4 h at pH 4.7 by the addition of 0.1 M HCl. Following reduction with sodium borodeuteride (100 mg, 4 h), the neutralized (dilute AcOH) product was dialyzed against running water and the retentate was lyophilized. The product was subjected to a second reduction under the same conditions and the final reduced O-PS (81 mg) was obtained as the lyophilized void volume product obtained on Sephadex G-50 gel-filtration chromatography.

3. Results and discussion

E. coli O48:H21 (NRCC 6366) was fermenter grown in 3.7% brain–heart infusion at 37 °C and the collected cell mass was extracted by a modified hot aqueous phenol procedure.[19] LPS was found almost exclusively in the separated aqueous phase and was obtained by ultracentrifugation. Hydrolysis of the LPS with hot dilute acetic acid afforded an insoluble lipid A (22%), and Sephadex G-50 column gel-filtration of the water-soluble product gave a void volume O-polysaccharide (O-PS, K_{av} 0.04, 35% yield), which by colorimetric analysis was indicated to be composed of neutral aldose, 2-amino-2-deoxyhexose, and uronic acid in an approximate 2:2:1 molar ratio.

The O-PS had $[\alpha]_D$ +95 (*c* 2.5, water). The O-PS hydrolysis product (2 M TFA, 105 °C, 2 h) analyzed by the GLC–MS alditol acetate method[20] was indicated to be composed of rhamnose (Rha), galactose (Gal), 2-amino-2-deoxyglucose (GlcN), and 2-amino-2-deoxygalactose (GalN) in approximately equimolar ratios. The hexuronic acid O-PS component was subsequently identified as galacturonic acid (GalA) from the GLC–MS characterization of galactitol-1-*d*,6-d_2 in the hydrolysis product of the carboxyl reduced ($NaBD_4$) O-PS obtained using the carbodiimide reduction procedure.[18] GLC analysis of the 2-(+)-butyl glycosides[14] and the determination of the specific optical rotation of the individual glycose components, obtained by preparative paper chromatography, established their identification as L-Rha, D-Gal, D-GlcN, D-GalN, and D-GalA. DOC-PAGE analysis of the LPS (Fig. 1) showed a

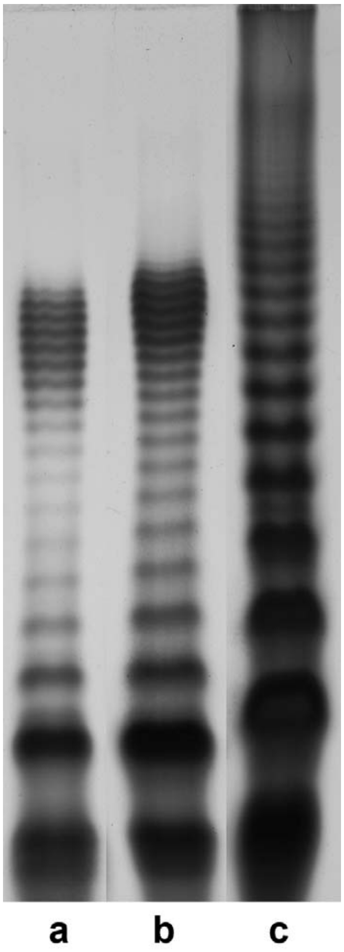


Figure 1. DOC-PAGE of the LPS produced by *E. coli* O48:H21. Lanes (a) and (b) *E. coli* O48 LPS, lane (c), reference LPS from *Salmonella milwaukee*[27] (O-PS pentasaccharide repeating unit).

typical ladder banding pattern with a between step spacing indicative of an O-PS composed of repeating pentasaccharide units.[21]

Since the NMR spectra of the isolated O-PS showed minor heterogeneity owing to non-stoichiometric O-acetylation (CH_3CO, <3% total mass), a portion of O-PS was O-deacetylated by treatment with dilute ammonium hydroxide to give an *O*-acetyl free product on which all further analyses were made. The new O-PS had $[\alpha]_D$ +87 (*c* 2.0, water). Anal. C, 36.80; H, 5.8; N, 3.13; and ash, ~2%. The ^{1}H NMR spectrum (Fig. 2) showed inter alia, five anomeric proton signals at 5.23 ($J_{1,2}$ 2.8 Hz), 5.02 ($J_{1,2} \sim 3$ Hz), 4.86 ($J_{1,2}$ 7.6 Hz), 4.85 ($J_{1,2}$ nr), and 4.65 ppm ($J_{1,2}$ 7.8 Hz) consistent with the proposed pentasaccharide unit. As expected, two *N*-acetyl resonances at 2.12 (3H) and 1.98 ppm (3H), and one methyl signal at 1.30 ppm (3H) characteristic of the detected L-Rha residue were also present. The corresponding ^{13}C NMR spectrum of the O-deacetylated O-PS (Fig. 3a) showed inter alia, five anomeric resonances at 103.8 ($J_{C\text{-}1,H\text{-}1}$ 164 Hz), 102.1 ($J_{C\text{-}1,H\text{-}1}$ 173 Hz), 102.0 ($J_{C\text{-}1,H\text{-}1}$ 165 Hz), 100.8 ($J_{C\text{-}1,H\text{-}1}$ 174 Hz), and 96.3 ppm ($J_{C\text{-}1,H\text{-}1}$ 174 Hz), with $J_{C\text{-}1,H\text{-}1}$ couplings[22] indicative of two β- and three α-glycosidic linkages in the O-PS repeating unit. Also present in the spectrum was a methyl singlet at 17.6 ppm characteristic of a 6-deoxyhexose (L-Rha), two sharp signals at 56.1 and 51.9 ppm characteristic of C–N links (deoxyaminohexose) and two methyl signals (CH_3CO–NH–) at 23.3 and 23.1 ppm and associated carbonyl signals at 175.2 and 175.4 ppm from acetamido substituents together with a third carbonyl signal at 176.1 ppm, assigned to the identified D-GalA constituent hexuronic acid.

The complete structural characterization of the O-PS was made from the results of two-dimensional NMR analysis involving COSY, TOCSY, NOESY, and ^{1}H–^{13}C HSQC and HMBC experiments, which were used to assign chemical shift spin systems of the five glycose residues present in a repeating unit (Fig. 2 and Table 1). The glycose residues in the proton NMR spectrum were labeled **A** to **E** in order of decreasing anomeric chemical shifts. Residue **A** was readily identified as an α-D-Gal*p*A hexuronic acid from the magnitude of its anomeric proton coupling constant ($J_{1,2}$ 2.8 Hz), and carbon coupling constant ($J_{H\text{-}1,C\text{-}1}$ 174 Hz), and from the correlation of its H-5**A** at 4.25 ppm to its carbonyl C-6**A** (176.1 Hz) seen in an HMBC experiment. An observed upfield shift of H-5**A** at 4.25 ppm (pD 8.5) to 4.51 ppm (pD 3.8) was seen together with an associated carbon (C-5**A**) shift from 73.1 to 71.9 ppm in the same pD adjustment and an associated shift of H-4**A** from 4.31 to 4.36 ppm was also seen with the pD change, characteristic of a free carboxyl group. Residue **C** was identified as a D-glucopyranose component from the large ring coupling constants (9.4–9.6 Hz) for $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$. Correlation of the H–2**C** (3.89 ppm) to the corresponding C–N shift at 56.1 ppm in the carbon axis taken with consideration of the coupling constant ($J_{1,2}$ 7.6 Hz) measured in 2D homonuclear COSY, together with the anomeric coupling constants measured from the carboxyl reduced O-PS ($J_{C\text{-}1,H\text{-}1}$ 165 Hz), allows **C** to be identified as a

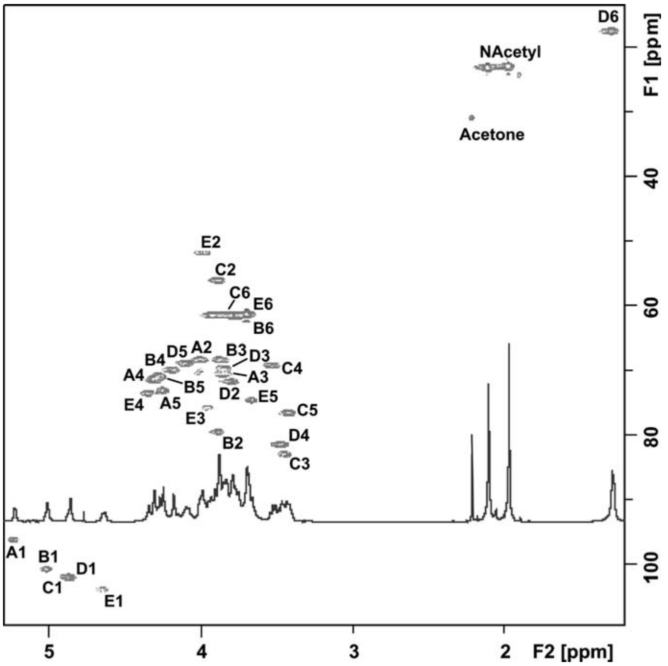


Figure 2. ^{1}H–^{13}C HSQC correlation spectrum of the O-deacetylated O-PS of *E. coli* O48:H21 showing proton and carbon correlated cross peaks for the component repeating unit glycoses [**A**] α-D-Gal*p*A-(1→, [**B**] →3)-α-D-Gal*p*-(1→, [**C**] →3)-β-D-Glc*p*NAc-(1→, [**D**] →4)-α-L-Rha*p*-(1→, and [**E**] →3,4)-β-D-Gal*p*NAc-(1→, together with the 1D ^{1}H NMR spectrum.

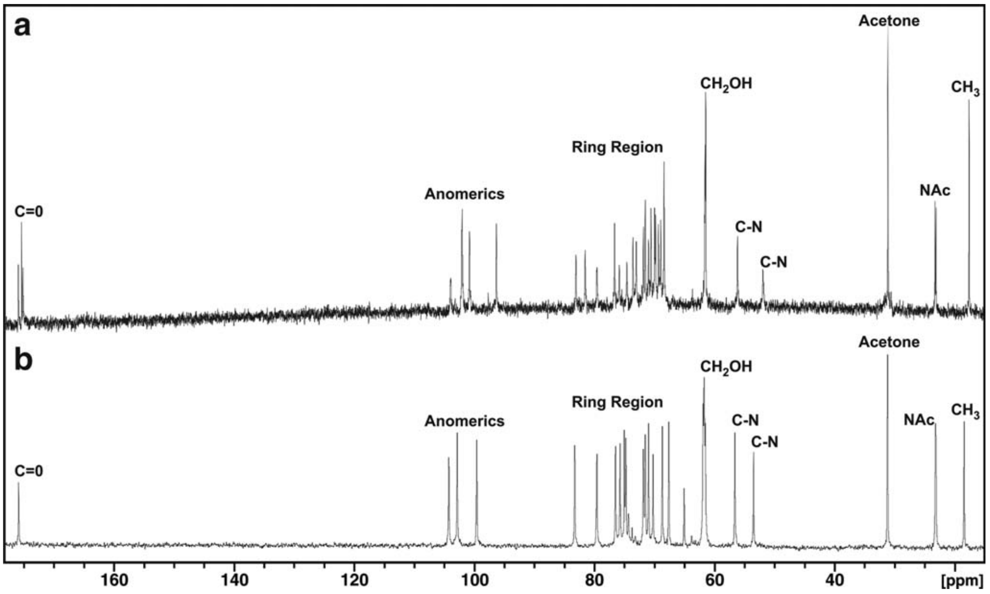


Figure 3. ^{13}C NMR spectra of (a) O-deacetylated *E. coli* O48:H21 O-PS and (b) derived oligosaccharide **I** from O-PS.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of *Escherichia coli* serotype O48:H21 O-deacetylated O-PS with inter-residue correlations made from NOESY and HMBC experiments

Residue	^{1}H/^{13}C chemical shifts (ppm)							
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H5/C-5	H6/C-6	Connectivity from anomeric H-1	
							NOE	HMBC
A α-D-Gal*p*A-(1→	5.23 (2.8)	4.01	3.85	4.31	4.25	—		76.0; C-3**E**
	96.3 (174)	68.5	70.5	71.5	73.1	176.1		
B →3)-α-D-Gal*p*-(1→	5.02 (∼3)	3.88	3.89	4.18	4.28	3.70	3.48; H-4**D**	81.5; C-4**D**
	100.8 (174)	68.3	79.6	70.0	70.9	61.8		
C →3)-β-D-Glc*p*NAc-(1→	4.86 (7.6)	3.89	3.45	3.53	3.43	3.9/3.8	4.35; H-4**E**	
	102 (165)	56.1	83.1	69.3	76.6	61.6		
D →4)-α-L-Rha*p*-(1→	4.85 (∼nr)	3.80	3.84	3.48	4.10	1.30	3.45; H-3**C**	83.1; C-3**C**
	102.1 (173)	71.8	69.8	81.5	68.9	17.6		
E →3,4)-β-D-Gal*p*NAc-(1→	4.65 (7.8)	3.97	3.95	4.35	3.67	3.76/3.8		79.6; C-3**B**
	103.8 (164)	51.9	75.8	73.5	74.5	61.6		

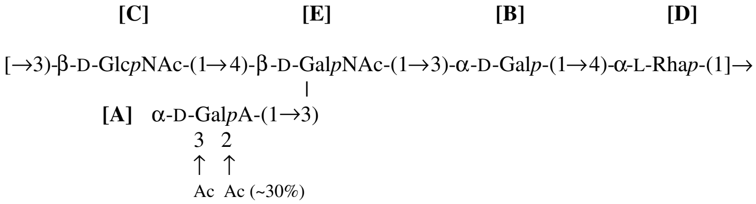
Spectra were recorded in D$_2$O at 400 MHz and $J_{1,2}$ and $J_{\text{C-1,H-1}}$ values given in hertz (in parentheses) are calculated from 2D NMR.

β-D-Glc*p*NAc residue. **E** was identified as a β-D-Gal*p*NAc residue from its anomeric coupling constants ($J_{1,2}$ 7.8; $J_{\text{C-1,H-1}}$ 164 Hz) and from a correlation of H-2**E** (3.97 ppm) to the carbon shift C-2**E** (51.9 ppm) characteristic of a nitrogen bearing carbon (C–N). Residue **B** was assigned a *galacto* configuration from a consideration of its coupling constants ($J_{2,3}$ 9.8, $J_{3,4}$ 3.8, and $J_{4,5} \sim 1$ Hz) and as an α-D-Gal*p* component from its anomeric coupling constants ($J_{1,2} \sim 3$, and $\text{J}_{\text{C-1,H-1}}$ 174 Hz). Residue **D** was identified as α-L-Rha*p* ($J_{\text{C-1,H-1}}$ 173 Hz) from its characteristic coupling constants.

Methylation of the O-deacylated **O**-PS followed by its hydrolysis and reduction (NaBD$_4$) gave a mixture of methylated glycitols, which were acetylated and analyzed by GLC–MS. The following derivatives were identified: 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylrhamnitiol-1*d* (T_{GM} 0.87), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol-1*d* (T_{GM} 1.40), 1,3,5-tri-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-4,6-di-*O*-methylglucitol-1*d* (T_{GM} 2.60), and 1,3,4,5-tetra-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-6-*O*-methylgalactitol-1*d* (low yield, T_{GM} 3.21). In a separate analysis a portion of the methylated **O**-PS was carboxyl reduced (NaBD$_4$–THF), and by GLC–MS analysis, as described, showed the above methylated derivatives together with 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol-1*d*,6*d*$_2$ (T_{GM} 1.56) derived from the D-GalA residue, and an increased yield of 1,3,4,5-tetra-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-6-*O*-methylgalactitol-1*d*.

The methylation evidence is consistent with the NMR conclusions and confirms that residue **A** is an unsubstituted galactopyranosyluronic acid residue, **B** is a 3-O-substituted galactose, **C** is a 3-O-substituted 2-acetamido-2-deoxyglucose, **D** is a 4-O-substituted rhamnose, and **E** is a 3,4-di-O-disubstituted 2-acetamido-2-deoxygalactose residue.

In the NOESY-NMR spectrum of the O-deacetylated O-PS (Table 1), NOE's were seen from residue **B** (α-D-Gal*p*) H-1 to H-4 of **D** (α-L-Rha*p*), from residue **D** (α-Rha*p*) to H-3**C**, from residue H-1**C** (β-D-Glc*p*NAc) to H-4 of residue **E** (β-D-Gal*p*NAc). HMBC evidence showed inter-residue correlations from the anomeric protons of **A** → C-3**E**, **E** → C-3**B**, **B** → C-4**D**, **D** → C-3**C**. A consideration of the composition, methylation, and NMR data is consistent with the basic repeating unit of the O-PS having the structure:



[C] [E] [B] [D]

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

[A] α-D-Gal*p*A-(1→3)

3 2

Ac Ac (~30%)

$$[\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1]\rightarrow$$

The location of the acetyl groups at O-2 and O-3 of the D-GalA residue and their degree of substitution were determined from the approximate quantitation of the resonances at H-2 (4.01 ppm) and H-3 (3.85 ppm) of the D-Gal*p* residue in the ^{1}H NMR spectrum of the O-deacetylated O-PS, in comparison with the corresponding resonances present in the spectrum of the isolated O-PS.

A confirmation of the proposed O-PS structure was obtained from the analysis of the oligosaccharide obtained on a Smith type[16] degradation of the carboxyl reduced O-PS. The periodate oxidized and reduced ($NaBH_4$) O-PS polymer product was hydrolyzed under mild conditions and the major oligosaccharide obtained on Biogel P2 column chromatography (K_{av} 0.35) on GLC analysis by the alditol acetate procedure showed peaks identified as the acetate derivatives of galactitol (T_G 1.01), 2-amino-2-deoxyglucitol (T_G 1.22), 2-amino-2-deoxygalactitol (T_G 1.25), and 1-deoxyerythritol (T_G 0.15) (1:1:1:1). The ^{1}H NMR spectrum (Table 2) of the oligosaccharide showed three anomeric signals at 5.10 ($J_{1,2}$ 2.8 Hz), 4.72 ($J_{1,2}$ 8.5 Hz), and 4.63 ppm ($J_{1,2}$ 8.2 Hz), corresponding, respectively, with carbon shifts (Fig. 3b) located at 103.7 ($J_{\text{C-1,H-1}}$ 165 Hz), 102.3 ($J_{\text{C-1,H-1}}$ 165 Hz), and 99.1 ppm ($J_{\text{C-1,H-1}}$ 172 Hz). 2D NMR analysis showed resonances at 56.0 and 52.9 ppm together with signals at 22.4 and 22.5 ppm associated with two carbonyl resonances at 174.8 and 174.9 ppm, respectively, arising from C*H*–N and CH_3 and CH_3*C*O–N groups of the periodate resistant D-Glc*p*NAc and D-Gal*p*NAc components. The presence of a single methyl signal at 1.24 ppm (3H) arises from a terminal 1-deoxyerythritol residue derived from the periodate oxidation of the 1,4 linked α-L-Rha*p* residues in the O-PS. The oligosaccharide on ESIMS analysis in the negative ion mode gave an expected molecular ion $[M+OH]^- = 674$. The results of the above analyses identified the oligosaccharide as having the structure:

[G] [H] [F] [I]

β-D-Glc*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→3)-1-deoxy-D-erythritol

The characterization of the above oligosaccharide was consistent with the proposed O-PS pentasaccharide repeating unit structure. In particular, the characterization of 2-deoxy-2-(*N*-methylamino)-3,6-di-*O*-methylgal-

Table 2. ^{1}H and ^{13}C NMR chemical shifts (ppm) of oligosaccharide **I** derived from the Smith type periodate oxidation of the O-deacetylated O-PS of *Escherichia coli* O48:H21

Residue	^{1}H/^{13}C chemical shifts (ppm)					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
F →3)-α-D-Gal*p*-(1→	5.10 (2.8)	3.87	3.97	4.16	4.13	3.72
	99.1 (172)	68.1	79.0	69.6	70.9	61.1
G β-D-Glc*p*NAc-(1→	4.72 (8.5)	3.75	3.58	3.44	3.44	3.93/3.76
	102.3 (165)	56.0	74.2	70.4	75.9	61.0
H →4)-β-D-Gal*p*NAc-(1→	4.63 (8.2)	3.86	3.86	4.10	3.67	3.78
	103.7 (165)	52.9	71.3	75.2	74.4	61.6
I →3)-1-Deoxy-D-erythritol	1.24	4.05	3.65	3.78/3.80		
	17.7	67.0	82.7	61.6		

Spectra were recorded in D_2O at 400 MHz and $J_{1,2}$ and $J_{\text{C-1,H-1}}$ values given in hertz (in parentheses) are calculated from 2D NMR.

actitol in the hydrolysis products of the methylated oligosaccharide demonstrated its 4-O-linked glycosyl substitution in the linear backbone structure and confirmed its 3-O-substitution by single Gal*p*A (**A**) residues in the native O-PS.

It is not unusual to find that O-antigens of *E. coli* are produced in the LPS of unrelated bacterial pathogens. For example, in the *E. coli* O157:H7 EHEC strain the O-antigen[3] is chemically and serologically identical with the LPS O-antigen of *Salmonella* serogroup N (O:30),[23] *Citrobacter szelaki* and *C. freundii* isolates,[24] and relationships of their O-antigen gene clusters have suggested common ancestor origin.[25] It is interesting to note that the same basic pentasaccharide repeating unit now found present in the O-PS of *E. coli* O48 was also found in the O-PS produced by *Edwardsiella tarda* MT,[26] a Gram-negative bacterium that is an important cause of hemorrhagic septicemia in fish and also gastro- and extraintestinal infections in humans.[26] In the *E. tarda* O-PS, the α-D-Gal*p*A residue carboxyl group was, however, present in amidation with (2*S*,3*R*)-threonine.

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