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Structural elucidation of the O-antigenic polysaccharide from the enteroaggregative *Escherichia coli* strain 180/C3 and its immunochemical relationship with *E. coli* O5 and O65

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Abstract—The structure of the O-antigen polysaccharide (PS) from the enteroaggregative *Escherichia coli* strain 180/C3 has been determined. Sugar and methylation analysis together with ^{1}H and ^{13}C NMR spectroscopy were the main methods used. The PS is composed of tetrasaccharide repeating units with the following structure:

→2)-β-D-Qui*p*3NAc-(1→3)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→

Analysis of NMR data indicates that the presented sequence of sugar residues also represents the biological repeating unit of the O-chain. The structure is closely related to that of O-antigen polysaccharide from *E. coli* O5 and partially to that of *E. coli* O65. The difference between the O-antigen from the 180/C3 strain and that of *E. coli* O5 is the linkage to the D-Qui*p*3NAc residue, which in the latter strain is 4-*O*-substituted. The *E. coli* O65 O-antigen contains as part of its linear pentasaccharide repeating unit a similar structural element, namely →4)-β-D-Gal*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Qui*p*3NAc-(1→, thereby indicating that a common epitope could be present for the two polysaccharides. Monospecific anti-*E. coli* O5 rabbit serum did not distinguish between the two positional isomeric structures neither in slide agglutination nor in an indirect enzyme immunoassay. The anti-O65 serum did react with both the 180/C3 and O5 LPS showing a partial cross-reactivity.

Keywords: *Escherichia coli*; Enteroaggregative; Serology; Lipopolysaccharide; NMR

1. Introduction

Diarrheagenic *Escherichia coli* strains are major pathogens associated with enteric disease in many parts of the world. Currently, five pathotypes of diarrheagenic *E. coli* have been unequivocally associated with diarrheal illness: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), verocytotoxin-producing *E. coli* (VTEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAggEC).[1] The latter may be an emerging diarrheal pathogen. This pathotype, defined by aggregative adherence to Hep-2 cells in culture, has been associated characteristically with persistent diarrhea among infants, particularly in the developing

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world.[2–6] However, recent outbreaks and volunteer studies suggest that EAggEC strains are virulent in adults[7,8] and have a global distribution.[9,10] In a recent publication, Czeczulin et al.[11] analyzed different EAggEC and diffusely adherent *E. coli* (DAEC) by multilocus enzyme electrophoresis and showed that a majority of EAggEC strains could be divided into two closely related but distinct groups. Both groups were easily separated from other pathotypes of *E. coli*. It is not known whether there are any correlations between the phylogenetic grouping of the EAggEC strains and the structure of the O-antigens. The strain 180/C3 used in this study could not be serotyped by conventional methods because of its auto-agglutinating property. We have therefore undertaken the structural analysis of the O-antigenic polysaccharide isolated from the lipopolysaccharide (LPS) of the *E. coli* strain 180/C3. Once the structure had been elucidated, it was shown to be very similar to the O-antigenic polysaccharides from *E. coli* O5 and partially similar to that of *E. coli* O65. The relationships were also evident from immunochemical experiments presented herein.

2. Results and discussion

The *E. coli* strain 180/C3 was grown in a glucose containing tryptone/yeast extract medium. The LPS was isolated from the bacterial membrane by hot phenol/water extraction and delipidated under mild acidic conditions to yield a polysaccharide (PS). A hydrolysate of the PS contained ribose, 3-amino-3,6-dideoxyglucose (Qui3N), galactose, and 2-amino-2-deoxygalactose in the ratio 1.5:1.0:3.1:2.3. Glucose and heptose were also detected but these components were attributed to the core. Determination of the absolute configuration of the above four components revealed that they had the D-configuration. Methylation analysis showed the presence of four components, namely, 2,5-di-*O*-methyl-ribose, 2,3,6-tri-*O*-methyl-galactose, 3,6-dideoxy-3-*N*-methylacetamido-4-*O*-methyl-glucose, and 2-deoxy-2-*N*-methylacetamido-4,6-di-*O*-methyl-galactose in the ratio 1.2:2.3:1.1:1.0.

The ^{1}H NMR spectrum showed the presence of a signal from a methyl group (δ 1.34, J = 6.0 Hz) and methyl signals from *N*-acetyl groups (δ 2.06 and 2.07, 6H) revealing that the amino sugars are *N*-acetylated. In the region for anomeric resonances five signals were present, four of which could be shown to originate from anomeric protons since the ^{13}C NMR spectrum showed four resonances from anomeric carbons in the region δ 96.3–109.3. Thus, the PS consists of tetrasaccharide repeating units. The ^{1}H and ^{13}C NMR spectra were assigned using two-dimensional NMR techniques and the chemical shifts are compiled in Table 1.

The resonance at δ_H 5.38 had $J_{H1,H2}$ = 3.8 Hz and its C-2 resonance at δ_C 48.6. From methylation analysis a pyranoid ring form can be deduced. These results show the residue to be an α-linked *N*-acetylgalactosamine. The ^{13}C NMR glycosylation shifts[12] reveal, together with the above methylation analysis, the substitution pattern of the sugar residues. Thus, residue **A** is 3-substituted. The anomeric proton resonance of residue **B** at δ_H 5.26 had $J_{H1,H2}$ = 2.0 Hz. The anomeric carbon resonance was observed at δ_C 109.3, indicative of a β-linked

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the signals from the O-antigen polysaccharide of the *Escherichia coli* strain 180/C3 and inter-residue correlations from NOESY and HMBC spectra

Sugar residue	^{1}H/^{13}C							
	1	2	3	4	5	6	Connectivity to atom (from anomeric atom)	
							NOE (δ_H)	HMBC (δ_H/δ_C)
→3)-α-D-Gal*p*NAc-(1→[a]	5.38 [3.8]	4.41	3.74	4.21	3.86	~3.79	3.59; **C**, H2	
A	(0.10)	(0.22)	(−0.21)	(0.16)	(−0.27)			
	96.3 {178}	48.6	78.4	69.5	71.8	61.6		3.59; **C**, H2
	(4.3)	(−2.6)	(10.0)	(−0.1)	(0.4)	(−0.5)		
→3)-β-D-Rib*f*-(1→	5.26 [2.0]	4.30	4.27	4.09	3.62, 3.72		4.03; **D**, H4	76.6; **D**, C4
B	109.3 {177}	75.3	78.9	81.7	63.1			4.03; **D**, H4
	(7.6)	(−0.7)	(7.7)	(−1.6)	(−0.2)			
→2)-β-D-Qui*p*3NAc-(1→[b]	4.76 [7.6]	3.59	3.95	3.29	3.59	1.34	4.27; **B**, H3	78.9; **B**, C3
C	103.5 {164}	73.6	56.3	73.6	74.1	17.8		4.27; **B**, H3
→4)-β-D-Gal*p*-(1→	4.44 [7.6]	3.53	3.75	4.03	3.73	3.74, 3.77		78.4; **A**, C3
D	(−0.09)	(0.08)	(0.16)	(0.14)	(0.08)			
	105.9 {162}	71.5	73.6	76.6	75.3	61.9		3.74; **A**, H3
	(8.5)	(−1.5)	(−0.2)	(6.9)	(−0.6)	(−0.1)		

$J_{H1,H2}$ values are given in hertz in square brackets and $J_{H1,C1}$ values in braces. Chemical shift differences as compared to the corresponding monosaccharides are given in parenthesis.

[a] Chemical shifts for NAc are δ_H 2.06; δ_C 23.1 and 174.8.

[b] Chemical shifts for NAc are δ_H 2.07; δ_C 23.3 and 175.3.

furanosidic ring form. Disentangling of the spin system showed that it belonged to the ribosyl residue and subsequently it is a β-D-Rib*f* residue. In support of this a correlation between H1 and C4 was present in the ^{1}H, ^{13}C HMBC spectrum, a finding previously observed for galactofuranosides.[13] The carbon-13 glycosylation shift shows that residue **B** is 3-substituted. Residue **C** has its anomeric proton resonance at δ_H 4.76 and showed $J_{H1,H2}$ = 7.6 Hz. Unraveling of the proton spin system unambiguously revealed it as the β-D-Qui*p*3NAc residue, which consequently is 2-substituted. Finally, residue **D** with its anomeric proton resonance at δ_H 4.44 and $J_{H1,H2}$ = 7.6 Hz could be assigned to a 4-substituted β-D-Gal*p* residue.

The sequence of the sugar residues in the repeating unit was determined from $^{1}H,^{1}H$ NOESY and $^{1}H,^{13}C$ HMBC experiments. The connectivities are also given in Table 1, from which the sequence –**C**–**B**–**D**–**A**– can be deduced. Thus, the structure of the O-antigen polysaccharide from *E. coli* strain 180/C3 is:

C **B** **D** **A**

→2)-β-D-Qui*p*3NAc-(1→3)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→

Analysis of minor peaks in the NMR spectra revealed spin systems of sugar residues similar to those of the repeating units of the O-polysaccharide. These data indicate that sugar residues in the terminal repeating unit (denoted by a prime) could be identified, namely, at δ_{H1} 4.59 ($J_{H1,H2} \sim 8$ Hz) and δ_{C1} 103.9 for **C**′, δ_{H1} 5.31 and δ_{C1} 109.2 for **B**′, δ_{H1} 4.48 ($J_{H1,H2} \sim 8$ Hz) and δ_{C1} 105.6 for **D**′. In 2D NMR spectra correlations were observed, inter alia, between C1 in **C**′ and H2 in **C**′ (3.38 ppm, cf. Table 1), indicating a terminal β-D-Qui*p*3NAc residue, as well as H1 in **C**′ and C3 in **B**′ (79.5 ppm) in the $^{1}H,^{13}C$ HMBC spectrum, and between H1 in **B**′ and H3 in **D**′ (4.03 ppm) in the $^{1}H,^{1}H$ NOESY spectrum. In two recent determinations of *E. coli* O-antigens[14,15] a 3-substituted *N*-acetylglucosamine residue was present at the reducing end of the biological repeating unit. Based on the above NMR correlations, the structure of the repeating unit and biosynthetic considerations, an α-linked *N*-acetylgalactosamine residue should be present at the reducing end of the biological repeating unit. Further analysis using the CASPER program[16] also suggested that the sugar residue with δ_{H1} 4.80 ($J_{H1,H2} \sim 8$ Hz) and δ_{C1} 102.5 corresponds to a β-linked *N*-acetylgalactosamine residue (**A** at the reducing end of the O-chain), which substitutes the core of the LPS. Similarly, in the LPS from *Salmonella enterica* sv. Arizonae IIIa O62 there is a β-linkage between the O-chain and the core.[17] This is also the case in the LPS from *S. enterica* sv. Typhimurium strain 1135, although the corresponding sugar residues in the O-chain are α-linked.[18]

The structure of the O-antigenic polysaccharide present in the LPS from the enteroaggregative *E. coli* strain 180/C3 is very similar to the one present in *E. coli* O5.[19] The difference being the linkage between the aminosugars as shown below for the structure of the O-antigen polysaccharide from *E. coli* O5:

→4)-β-D-Qui*p*3NAc-(1→3)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→

The $^{1}H,^{13}C$ HSQC NMR spectra (Fig. 1) revealed that the polymers are indeed different. Thus, these O-antigenic polysaccharides are positional isomers. In addition, the 180/C3 O-polysaccharide has some structural elements in common with the *E. coli* O65 polysaccharide,[20] consisting of pentasaccharide repeating units with the following structure:

→2)-β-D-Qui*p*3NAc-(1→4)-α-D-Gal*p*AN-(1→4)-α-D-Gal*p*NAc-(1→4)-β-D-Gal*p*A-(1→3)-α-D-Glc*p*NAc-(1→

Initially, when the *E. coli* 180/C3 strain was tested in slide agglutination it showed to be auto-agglutinating. The strain was also O rough during the first screening using boiled culture in beef broth (normal saline) and required suspension in deionized water before the O group could be established as O5. In addition, the 180/C3 strain showed a weak reactivity with an antiserum against *E. coli* O65. In order to further investigate the similarity between the structures of the O-antigens present in the three strains, an indirect enzyme immunoassay

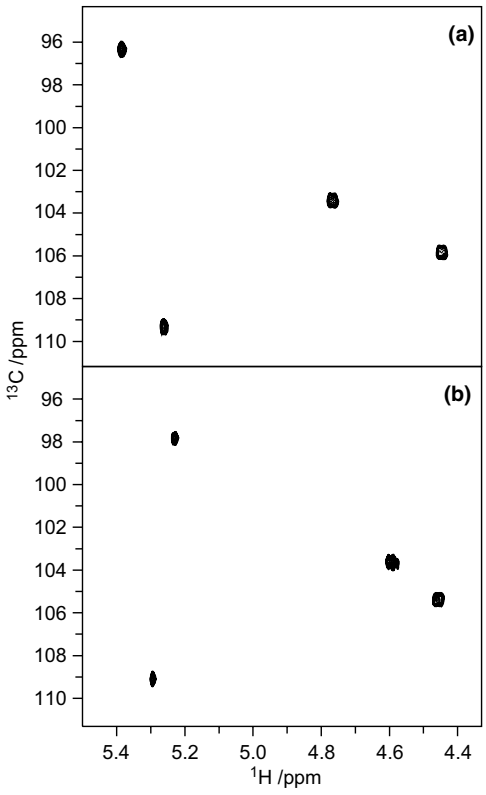

Figure 1. Anomeric region from the $^{1}H,^{13}C$ HSQC spectrum of the O-antigen PS from *E. coli* strain 180/C3 (a) and *E. coli* O5 (b).

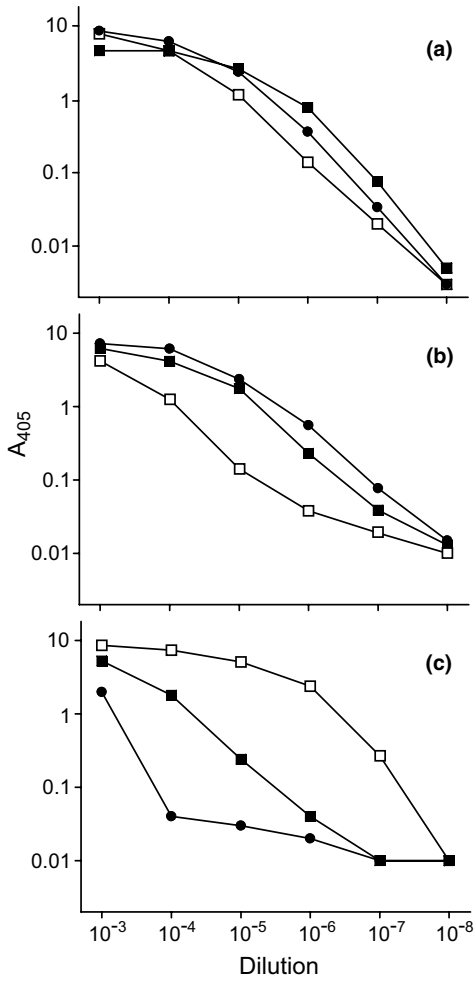

Figure 2. Enzyme immunoassay with rabbit anti-*E. coli* 180/C3 (■), O5 (●), and O65 (□) sera titrated against LPS isolated from *E. coli* strain 180/C3 (a), O5 (b), and O65 (c).

was used. Titration of the rabbit anti-*E. coli* 180/C3, O5, and O65 sera against the 180/C3 LPS showed essentially identical curves with the homologous and with the anti-O5 serum. The anti-O65 antibodies showed a slightly weaker response (Fig. 2a). However, when the O5 LPS was used as antigen, the reactivity of the anti-O5 and anti-180/C3 was almost identical but the anti-O65 much weaker (Fig. 2b). When the O65 LPS was used as coating antigen the partial cross-reactivities between the strains were even more pronounced (Fig. 2c). Thus, in these experiments, the anti-180/C3 as well as anti-O5 sera react similarly but differed from the anti-O65 sera.

These results show that agglutination with specific anti-O5-serum did not distinguish between strains when only a minor difference in the O-antigen polysaccharide structure is present. Neither did the direct enzyme immunoassay show any significant difference when specific anti-O5 serum was used. The close structural relationship between the O-antigens of the two strains and the fact that the *E. coli* 180/C3 strain could be typed as O5 with existing methodology does not warrant a designation of the O-antigen of *E. coli* 180/C3 as a new O-antigen within the existing scheme. There is, however, sufficient evidence for dividing the O5 group into subtypes O5ab and O5ac. The cross-reactivity between the *E. coli* O5 and O65 has been observed earlier[21] and absorbed antisera are still used in the standard routine operation procedure for O grouping of *E. coli*.

3. Experimental

3.1. Bacterial strain and conditions of growth

The *E. coli* strain 180/C3, was isolated in 1991 from an infant with persistent diarrhea in León, Nicaragua. The strain was nontypable by slide agglutination due to auto-agglutination or aggregation but typable as O5:H– by the routine procedures followed by The International *Escherichia* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark, which requires suspension of O rough (auto-agglutination in normal saline) bacterial antigen in deionized water before mixing with specific antiserum. In addition, the 180/C3 strain agglutinated weakly with a monospecific anti-*E. coli* O65 rabbit serum. The strain showed the typical

aggregative adherence pattern in a Hep-2 assay.[22–24] The *E. coli* O5:K4:H4 strain CCUG11307 was obtained from the Culture Collection University of Gothenburg, Sweden. The *E. coli* O65:K–:H– strain was obtained from The International *Escherichia* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark.

Bacteria were grown in submerged cultures to late exponential phase in 15 L of a tryptone/yeast extract medium[25] containing 1% glucose, using a 30-L fermentor (Belach AB, Stockholm, Sweden) under constant aeration at 37 °C and pH 7.0. A preculture (1.5 L) in the same medium was used to inoculate the fermentor. All cultures were checked for purity at the end of the growth cycle. The bacteria were killed with 1% (v/v) formaldehyde. After incubation 16 h at 4 °C the cells were separated from the media by continuous-flow centrifugation using a CEPA model LE centrifuge at a cylinder speed of 29,000*g* and a flow of 25 L h^{-1} (Carl Padberg Centrifugenbau, Lahr, Germany). The bacterial mass was then removed from the cylinder, washed once with NaCl/P_i (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 4 °C, 20 min), and finally re-suspended in distilled water.

3.2. Preparation of lipopolysaccharide and lipid-free polysaccharide

The lipopolysaccharide (LPS) was extracted by the hot phenol/water method.[26] The aqueous phase was dialyzed at 4 °C for 3–5 days against tap water, overnight against distilled water, concentrated under diminished pressure, and lyophilized. Contaminating nucleic acids were removed by ultracentrifugation (100,000*g*, 4 h, 4 °C). Lipid-free polysaccharide (PS) was prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100 °C for 5 h.[27] Lipid A was removed by centrifugation (10,000*g*, 20 min, 4 °C). The PS was further purified by gel-permeation chromatography.

3.3. Component analyses

The PS was hydrolyzed with 0.5 M trifluoroacetic acid at 100 °C for 16 h. After reduction with $NaBH_4$ and acetylation, the sample was analyzed by GLC. The absolute configuration of the sugars present in the PS was determined by derivation of the sugars as their acetylated (+)-2-butyl glycosides[28,29] or as their acetylated dithioacetals.[30,31] The methylation analysis was performed according to Hakomori[32] using sodium methylsulfinylmethanide and iodomethane in dimethyl sulfoxide. The methylated compounds were purified using Sep-Pak C_{18} cartridges (Millipore) and recovered using acetonitrile and ethanol.[33] The purified methylated sample was then hydrolyzed (2 M trifluoroacetic acid, 120 °C, 2 h), reduced with NaB^2H_4, and acetylated. The partially methylated alditol acetates were analyzed by GLC–MS.

3.4. GLC and GLC–MS analyses

Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused silica column (0.20 mm × 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C min^{-1} to 210 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett-Packard model 5890 series II gas chromatograph equipped with a flame ionization detector. The acetylated dithioacetals were analyzed isothermally at 280 °C on an Alltech EC-1 column (0.32 mm × 5 m). GLC–MS analysis was performed on a Thermo Quest GCQ plus spectrometer equipped with a CPSIL8CB fused silica column (15 m). A temperature program of 170 °C for 3 min followed by 3 °C min^{-1} to 250 °C was used with helium as carrier gas.

3.5. NMR spectroscopy

NMR spectra of the PS in D_2O were recorded at 45 °C using Varian Inova 400 and 600 MHz instruments. Chemical shifts are reported in ppm relative to internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propanoate (TSP, δ_H 0.00) or external 1,4-dioxan in D_2O (δ_C 67.4) as references. Data processing was performed using standard Varian VNMR software. ^1H, ^1H-correlated spectroscopy (COSY),[34] total correlation spectroscopy (TOCSY)[35] with mixing times of 30, 60, and 90 ms, gradient selected heteronuclear single quantum coherence (gHSQC),[36] and gradient selected heteronuclear multiple-bond correlation (gHMBC)[36,37] experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY)[38] experiment with a mixing time of 50 ms, and an HMBC experiment with a 60 ms delay for the evolution of long-range couplings were used. The chemical shifts were compared to those of the corresponding monosaccharides.[39,40]

3.6. Immunochemical analyses

The rabbit anti-*E. coli* O5, O65, and 180/C3 specific antisera used in this assay were obtained from The International *Escherichia* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark. The enzyme immunoassay (EIA) was performed as described previously.[41] Microtiter plates (Costar) were coated overnight at 20 °C with 100 μL of each LPS (5 μg mL^{-1}; 0.05 M sodium carbonate, pH 9.6). After washing with 0.15 M NaCl, 0.05% Tween 20, the antisera diluted in 0.01 M KP_i, 0.15 M NaCl, pH 7.4, 0.05% Tween 20 (NaCl/P_i/Tween) were added. After incubation for 4 h at 20 °C,

the plates were washed as above and incubated overnight at 20 °C with 100 μL of alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted in (NaCl/P_i/Tween). For developing, plates were washed as above, and 100 μL of a 1 M diethanolamine, 0.5 M $MgCl_2$, pH 9.8, 1 mg mL^{-1} sodium *p*-nitrophenol phosphate was added. Plates were incubated at 20 °C and read in a Multiskan Plus EIA reader (Labsystems) at 405 nm after 25, 50, and 100 min. The values were extrapolated to absorbance after 100 min of incubation.

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