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The relationship between the structures of the O polysaccharides from *Escherichia coli* O17 and O16

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Abstract

The chemical structure of the O16 antigen from the lipopolysaccharide of *Escherischia coli* strain P4 has been determined. Comparison with the structures of other O16 antigens and that of the O17 antigen explains the previously reported cross-reaction of O antigen from the O16 strain K-12 with anti-O17 antibody [D. Liu and P.R. Reeves, *Microbiology*, 140 (1994) 49–57]. © 1997 Elsevier Science Ltd.

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1. Introduction

Lipopolysaccharide produced by an *Escherichia coli* K-12 strain with restored *rfb* genes was shown [1] to contain O antigen that was serologically similar to the O16 antigen of strain P4 from the type culture collection. Restriction maps of DNA from the *rfb* gene cluster for *E. coli* K-12 and strain P4 were indistinguishable, so *E. coli* K-12 may be classified as an O16 strain. However, the K-12 strain cross-reacted with O17 antiserum, even though the O16 type strain P4 does not.

The O antigen from strain K-12 was found [2] to contain a glucose side branch which is thought not to be encoded in the *rfb* region. It was therefore suggested that the branch glucose might be absent from the O antigen of P4. The cross-reactivity to the O17 antibody might then be due to the presence of a branch glucose on the O17 antigen, which might have a main chain quite different from the O16 structure. Restriction maps for the *rfb* gene cluster from the O17 strain M493 showed no similarity to those obtained with material from O16 strains.

These hypotheses have been confirmed by the chemical structure of the P4 polysaccharide reported in this work and the structure of the O17 polysaccharide reported previously [3] and confirmed in the present investigation. The structure of the sugar backbone of the O16 antigen from strain P4 is the same as that reported previously for strains K-12 [2] and F11119-41 [4] but, unlike the O antigens from those strains, it is acetylated.

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2. Results

The structure of the O antigen of E. coli *strain P4.*—The O antigen, extracted from cells of strain P4 as described in the Experimental, consisted of *N*-acetylglucosamine, glucose, galactose and rhamnose. The ratio of these components, determined by Dionex chromatography following acid hydrolysis, was 1.0:0.9:0.4:0.5. The corresponding figures obtained [2] for the O antigen of *E. coli* K-12 were 1.0:1.8:0.7:0.6 and it was concluded that there was some destruction of rhamnose and galactose during hydrolysis. The unexpected destruction of galactose was previously ascribed to its being present in the furanose form [2]. The composition of the repeating unit of the polysaccharide from P4 was, therefore, *N*-acetylglucosamine, glucose, galactose, and rhamnose in the ratio 1:1:1:1.

This was confirmed by the ^{1}H NMR spectrum of *O*-deacetylated polysaccharide from P4, which contained four anomeric resonances of equal area. The chemical shifts for the anomeric protons, 4.89, 4.98, 5.00 and 5.09 ppm, were very close to those observed for four of the five anomeric protons of *O*-deacetylated antigen from strain K-12. The fifth signal in the spectrum for K-12 material was that assigned [2] to the anomeric proton of the branch glucose.

The chemical shifts for most of peaks in the ^{13}C NMR spectrum of *O*-deacetylated P4 polysaccharide (Table 1) were also similar to those for the main chain sugars in *O*-deacetylated K-12 antigen. The resonances assigned to the carbons of the side-chain glucose in the K-12 structure were absent and the C-6 and C-5 resonances of *N*-acetylglucosamine were shifted -5.4 and $+1.4$ ppm, as expected for removal of a glycosidic substituent from the 6-position. All the other signals were within 0.2 ppm of those in the spectrum of *O*-deacetylated K-12 antigen.

Both the ^{1}H and ^{13}C NMR spectra of the P4 polysaccharide before deacetylation were very complex as a consequence of non-stoichiometric acetyl substitution. The extent of substitution was estimated from the ^{1}H spectrum by comparing the total area of the acetyl peaks in the region between 2.0 and 2.2 ppm with the area of the rhamnose H-6 peaks between 1.2 and 1.4 ppm. An average of 2.5 ± 0.1 acetyl groups per repeat unit, of which 1.0 ± 0.1 were *N*-acetyl groups, having chemical shifts between 2.04 and 2.06 ppm.

The complexity of the spectrum indicated that the acetylation was heterogeneous and it was not possible to determine the positions of the *O*-acetyl substituents, other than that at O-2 of rhamnose. The proposed structure of the repeat unit in P4 polysaccharide (Fig. 1) is shown with acetate at an undetermined position.

The most probable locations, based on the available evidence, are sites on the *N*-acetylglucosamine. The heterogeneity was not introduced during preparation of the polysaccharide as the ^{1}H NMR spectrum of whole lipopolysaccharide was very similar to that for the isolated O antigen. The spectrum of lipopolysaccharide was relatively broad (Fig. 2), presumably as a consequence of aggregation, but the number and relative intensities of the acetate methyl peaks were the same.

Hydrolysis of the acetate groups was performed in the NMR tube by adding small quantities of trifluoroacetic acid (TFA) or sodium hydroxide. The additional acetates were removed before the 2-*O*-acetyl groups on rhamnose, and the *N*-acetyl groups were completely resistant to both acid and base under the conditions used. Unfortunately, the additional *O*-acetates all hydrolysed at the same rate and could not be distinguished from each other.

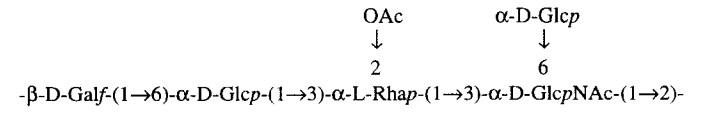
The relative peak intensities in the acetate methyl region of the ^{1}H NMR spectrum and their changes during deacetylation were consistent with the presence of *N*-acetylation of glucosamine (100%), *O*-acetylation of O-2 of rhamnose (site R, 100%) and

Table 1
^{13}C NMR chemical shifts for *O*-deacetylated P4 polysaccharide

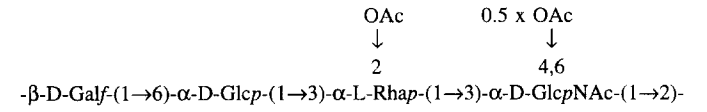
Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→ 2-β-Gal*f*	107.20	87.56	76.73	83.43	71.50?	64.15
→ 6-α-Glc*p*	98.17	72.50	74.14	70.16 [a]	71.50	66.98
→ 3-α-Rha*p*	102.04	69.18	78.11	71.58	70.56 [a]	17.80
→ 3-α-Glc*p*NAc	97.79	54.16	80.46	69.43	74.02	61.78

NAc 23.18.
[a] Assignments may need to be interchanged.

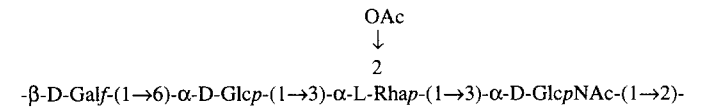
O16 K-12



O16 P4



O16 F11119-41



O17

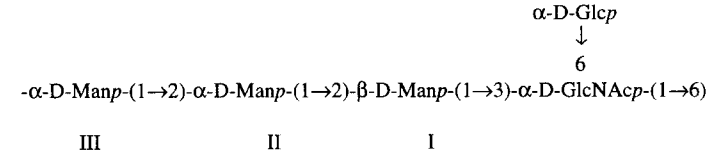


Fig. 1. Chemical structures of the repeat units from O16 and O17 antigens.

O-acetylation of two other sites (site A, ~ 30%; site B, ~ 25%). The presence of acetyl groups at sites A and B affected the chemical shifts of the *N*-acetyl methyl signal and that for the acetyl at site R. The chemical shift of the *N*-acetyl methyl signal was also split into two signals when the sample was hydrolysed to remove most of the *O*-acetyls, but leaving about half the 2-*O*-acetyl on rhamnose.

O-Acetyl substitution affected the rhamnose H-6 resonance as well, and at least seven doublets were observed. Two of the doublets were assigned using the spectra of partially deacetylated material. The doublet at 1.271 ppm was observed when only *N*-acetyl and rhamnose *O*-acetyl groups were present. That at 1.260 ppm was seen when only *N*-acetyl groups remained. The absence of the 1.260 ppm doublet in the spectrum of polysaccharide before hydrolysis confirmed that acetylation of the 2-position of rhamnose is complete in the original material.

The appearance of seven doublets suggests that there may be more than two additional acetylation sites, but a more economical interpretation is that the extra *O*-acetyl groups affect long-range interactions and can affect the conformation of neighbouring repeat units as well as the one in which they reside.

The ^{13}C NMR spectrum was similarly complex (Fig. 3). The acetate methyl region contained two signals not present in the spectrum for polysaccharide from strain K-12. Their intensities were roughly as expected for 25–30% substitution at sites A and B. The *N*-acetyl and rhamnose *O*-acetyl methyl signals were split into three peaks which collapsed into single lines after partial hydrolysis. As in the proton spectrum, there were more rhamnose C-6 signals (five) than the number of permutations of substituents at two sites. The number of carbonyl resonances (at least ten) was also greater than expected using the simplest model.

The region containing signals from the anomeric carbons suggested, if we assume that the acetylation

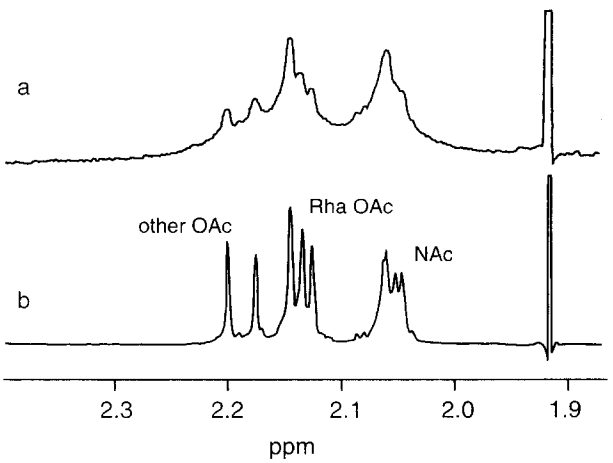


Fig. 2. Acetate methyl region of the 400-MHz ^{1}H NMR spectra of (a) lipopolysaccharide and (b) the O16 antigen from *E. coli* strain P4.

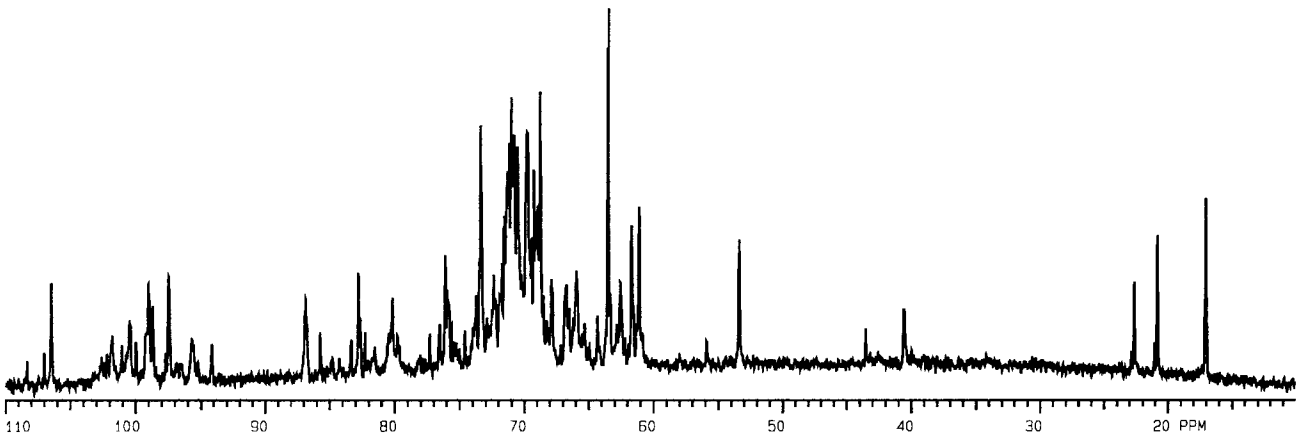


Fig. 3. 100-MHz ^{13}C NMR spectrum (carbonyl region omitted) of purified the O16 antigen from *E. coli* strain P4.

produces shifts of less than 5 ppm, that the C-1 signals for rhamnose and glucosamine were more severely affected than those for galactose, with glucose the least affected. Other identifiable signals, galactose C-2, C-3, and C-4; glucosamine C-3 and rhamnose C-3 were all affected by the additional *O*-acetylation.

Hence the O16 antigen of type strain P4 differed from the O antigen produced by *E. coli* K-12 by the absence of the side-chain glucose and the presence of an average of 0.5 acetyl groups per repeat unit at a minimum of two sites. These sites are probably on or near the *N*-acetylglucosamine residue. The only available sites on the *N*-acetylglucosamine are O-4 and O-6.

The structure of the O antigen of E. coli *O17.*— The O antigen was extracted from cells of the type O17 strain M493 as described in the Experimental. The normal procedure for monosaccharide analysis (2 M TFA, 100 °C, 4 h) resulted in incomplete hydrolysis and some disaccharide was observed by Dionex chromatography. More severe conditions (4 M HCl, 100 °C, 4 h) gave complete hydrolysis and released more glucosamine (relative to the internal standard 2-deoxy-D-*arabino*-hexose), but glucose and mannose were partially destroyed by the harsh conditions. The combined analyses were consistent with an overall composition of *N*-acetylglucosamine, glucose and mannose in the ratio 1:1:3, with 30% of a glucosamine–mannose disaccharide left uncleaved by the TFA hydrolysis.

The oligosaccharide was released by acetic acid hydrolysis as described in the Experimental. The structure of the oligosaccharide was determined by ^{1}H and ^{13}C NMR spectroscopy including COSY, HMBC, and ROESY correlation spectra. The anomeric configurations of the mannose rings were determined with the aid of $^{1}J_{CH}$ values and the ^{13}C NMR chemical shifts of the C-5 nuclei. Additional evidence for the linkage positions was obtained by methylation analysis. The results obtained were in complete agreement with those obtained independently by Masoud and Perry [3].

3. Discussion

The identical backbone structures of K-12, F11119-41 and P4 O16 antigens are consistent with their common interaction with standard O16 antiserum. An α-glucose is linked to the 6-position of *N*-acetylglucosamine in both O17 and K-12 antigens, but not in the polysaccharides from the other two O16 strains. This pair of sugars is, therefore, responsible for the cross-reaction of K-12 with O17 antiserum.

All the transferases required for construction of the backbone structure of the O16 antigens, including the *O*-acetyl on the rhamnose residue, are believed to be encoded in the O antigen gene cluster, which was studied in detail in K-12 and shown by Southern blotting to be identical in P4 (2). The gene for the glucose transferase required for the side-chain glucose in K-12 antigen is thought to be elsewhere on the chromosome and the same must be true for the *O*-acetyl transferases responsible for the additional acetylation of P4 antigen, as the O antigen clusters in P4 and K-12 are the same as shown by Southern Hybridisation.

There are a number of possibilities for the site of interaction of the O16 antigens with O16 antiserum, but the *N*-acetylglucosamine residue alone can be

eliminated as it is present in the O17 structure. Furthermore, acetyl substitution on or near the *N*-acetylglucosamine in P4 polysaccharide did not noticeably affect the interaction. If it is true, as suggested by the NMR evidence, that acetylation can affect the tertiary structure of the polysaccharide, the expected reduction in the interaction may be even greater than 50%. On the balance of probabilities, part or all of the Gal*f*–Glc–Rha region of the repeat unit is the site of interaction.

The composition of the O17 antigen is similar to that of several *Salmonella* O antigens, which also consist of *N*-acetylglucosamine and three or four mannose residues. Despite the presence of a linkages that are common to both the O17 antigen and *Salmonella* O antigens that react with O6 and O7 antiserum, no reaction of O17 with commercial O6,7 antiserum was observed.

4. Experimental

Cell culture.—Cultures for lipopolysaccharide extraction were grown as before [1]. Yields were 335 g of wet cells for P4 and 520 g for M493.

Serology.—Anti *E. coli* O17 serum was from the Institute of Medical and Veterinary Science, Adelaide, Australia and anti-O6,7 of *Salmonella* group C1 was supplied by Difco (Detroit). Cross-reaction was tested by slide agglutination.

Preparation of polysaccharide.—Lipopolysaccharide was isolated from packed cells by phenol–water extraction [5] followed by nuclease treatment and ultracentrifugation three times (65,000 *g* overnight). The polysaccharide was prepared by hydrolysis of lipopolysaccharide (100 mg) in 1% AcOH (10 mL) at 100 °C for 90 min. Lipid A was removed by filtration through acid-washed Celite. The filtrate was freeze-dried, redissolved in water (0.25 mL), clarified by centrifugation and fractionated on a Bio-Gel P6 column (30 × 2.5 cm) eluted with water (0.5 mL/min). The eluate was monitored by refractive index and the high molecular weight fractions were pooled to give the polysaccharide.

Compositional analysis.—Polysaccharides (1 mg) were hydrolysed in 2 M TFA (0.2 mL) for 4 h at 100 °C and evaporated to dryness on a Savent Speed Vac concentrator. The composition was analysed by HPAE chromatography on a Dionex CarboPac PA1 column (4 × 250 mm) using isocratic elution with 15 mM NaOH and pulsed amperometric detection (Waters 464). Identification was by comparison with authentic standards.

Methylation analysis.—The polysaccharides were methylated as described by Ciucanu and Kerek [6]. Before extraction with CH_2Cl_2, the pH of the aq soln was adjusted to below 3. The methylated polysaccharide was hydrolysed in 2M TFA (0.5 mL) at 100 °C for 4 h. Reduction with NaB^2H_4 and acetylation was done conventionally [7]. The partially methylated alditol acetates were analysed by GC–MS (Fisons MD800) using a BP-X5 column (25 m × 0.25 mm, 0.25 μ film thickness). The oven temperature was held at 45 °C for 1 min, increased at 15 °C/min to 140 °C, increased at 4 °C/min to 300 °C and held for 10 min. The products were identified by their chromatographic properties and mass spectrometric fragmentation patterns [8].

Partial acid hydrolysis.—A soln of polysaccharide (20 mg) in D_2O (0.6 mL) was hydrolysed by the addition of 20 μL of 8 M TFA, followed by heating at 80 °C in an NMR tube. The hydrolysis was monitored by observation of the anomeric ^1H NMR peaks. After 2 h, the pH was adjusted to 7.0 with NaOH. Oligosaccharide fractions were eluted from a Bio-Gel P2 column (30 × 1 cm) with water (0.15 mL/min) and monitored using refractive index.

Periodate treatment.—To a soln of polysaccharide (1 mg) in 50 mM NaOAc buffer (pH 4.8, 200 μL) was added $NaIO_4$ (5 mg) and the mixture was stored in the dark at room temperature for 7 days. Ethylene glycol (5 μL) was added and the mixture stored for 8 h, desalted on a Bio-Gel P2 column (30 × 1 cm Bio-Rad) and freeze-dried. The mixture was hydrolysed in 2 M TFA (0.2 mL) at 100 °C for 4 h and the monosaccharide composition analysed as described above.

NMR spectroscopy.—All spectra except the HMBC spectrum were obtained in D_2O soln using a Varian XL-400 spectrometer. The HMBC spectrum was recorded on a Bruker AMX-600 spectrometer. The spectra of the O16 antigen were recorded at 30 °C while those for O17 were recorded at 60 °C to take advantage of the slightly better resolution at that temperature. ^1H chemical shifts are expressed relative to internal acetone at 2.230 ppm and ^{13}C chemical shifts relative to internal acetone at 31.45 ppm. Proton–proton correlation spectra (COSY) and proton–carbon correlation spectra (HETCOR) were performed using standard Varian software.

A 20-mg sample of P4 oligosaccharide in 0.6 mL D_2O was progressively hydrolysed in the NMR tube by adding 3, 6 and 9 μL of TFA followed by 30 min incubation at 60 °C, after which time, no further changes in the NMR spectrum were observed. A

5-mg sample was hydrolysed by adding 20 μL of 1 M NaOD in D_2O.

Acknowledgements

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