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Structure of the β-L-fucopyranosyl phosphate-containing O-specific polysaccharide of *Escherichia coli* O84

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ABSTRACT

Fine structure of the O-polysaccharide chain of the lipopolysaccharide (O-antigen) defines the serospecificity of bacterial cells, which is the basis for O-serotyping of medically and agriculturally important gram-negative bacteria including *Escherichia coli*. In order to obtain the O-polysaccharide for structural analysis, the lipopolysaccharide was isolated from cells of *E. coli* O84a by phenol/water extraction and degraded with mild acid. However, the O-polysaccharide was cleaved at a highly acid-labile β-L-fucopyranosyl phosphate (β-L-Fuc*p*-1-*P*) linkage to give mainly a pentasaccharide that corresponded to the O-polysaccharide repeat. Therefore, the lipopolysaccharide and the pentasaccharide as well as their O-deacylated derivatives were studied using sugar analysis, NMR spectroscopy, and (for oligosaccharides) ESI HR MS, and the O84-polysaccharide structure was established. The O-polysaccharide is distinguished by the presence of β-L-Fuc*p*-1-*P* and randomly di-O-acetylated 6-deoxy-D-talose, which are found for the first time in natural carbohydrates. The gene cluster for the O84-antigen biosynthesis was analysed and its content was found to be consistent with the O-polysaccharide structure.

1. Introduction

Escherichia coli is a clonal species comprising both commensal and pathogenic strains. Harmless strains are part of the normal flora of the gut and can benefit their hosts by preventing colonization of the intestine with pathogenic microorganisms. However, some strains are virulent and can cause gastroenteritis, urinary tract infections, and neonatal meningitis. Certain strains of *E. coli* are a major cause of foodborne illness. Recently, strain O104:H4 was the subject of a serious bacterial outbreak that began in Germany. A Shiga toxin-producing strain O157:H7 causes hemorrhagic colitis and hemolytic-uremic syndrome.

There are more than 180 O-serogroups of *E. coli*, each expressing a distinct O-antigen. Aiming at creation of the chemical basis for classification of *E. coli* strains and elucidation of their evolution history, structures of the O-antigens have been established for most of the O-serogroups (http://www.casper.organ.su.se/ECODAB/) but some remain unknown. The O-antigen represents the O-specific polysaccharide chain of the lipopolysaccharide (LPS), which is localized on the cell surface of gram-negative bacteria. It consists of many oligosaccharide repeats (O-units) and is linked to the lipid moiety of the LPS (lipid A) via a large core oligosaccharide. The O-polysaccharides are built up of hexoses, 6-deoxyhexoses, amino and diamino sugars, uronic or nonulososnic acids and may also contain various non-sugar components. Several O-polysaccharides of *E. coli* are phosphorylated and a few include a glycopyranosyl phosphate linkage in the main chain [1–5].

The different O-antigen forms are mainly due to variations in the gene cluster for O-antigen biosynthesis, which is localized between conserved *galF* and *gnd* genes on the chromosome. The cluster usually contains (i) genes for synthesis of nucleotide precursors of specific monosaccharide components of the O-polysaccharide, (ii) glycosyltransferase genes for the O-unit assembly by sequential sugar transfer, and (iii) O-antigen processing genes, including *wzx* for O-unit flippase (translocase) and *wzy* for O-antigen polymerase.

Abbreviations: 6dTal, 6-deoxytalose; CID, collision-induced dissociation; COSY, correlation spectroscopy; DOS, O-deacetylated OS; ESI HR MS, electrospray ionization high-resolution mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; LPS, lipopolysaccharide; LPS_{OH}, O-deacylated LPS; OS, a mixture of oligosaccharides; ROESY, rotating frame overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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In this work, we studied structure and genetics of biosynthesis of the O-antigen *E. coli* O84, which has not been investigated yet. Strains of the O84 serogroup belong to Shiga toxin-producing *E. coli* (STEC), which are widespread in cattle and sheep populations and associated with diarrhea and/or hemolytic-uremic syndrome in humans [6,7]. The O84-polysaccharide was found to be a novel phosphorylated O-polysaccharide containing β-L-fucopyranosyl phosphate. The O84-antigen gene cluster was also characterized and was consistent with the O-polysaccharide structure.

2. Materials and methods

2.1. Growth of bacteria and isolation of the lipopolysaccharide

Cells of *E. coli* O84 type strain (laboratory stock numbers G1205) from the Institute of Medical and Veterinary Science, Adelaide, Australia (IMVS) was cultivated to late log phase in 8 L of Luria Bertani broth under constant aeration at 37 °C and pH 7.0 and stirring. Bacterial cells were washed and dried as described [8]. Cells were extracted using the phenol/water procedure [9], and the crude material was recovered by dialysis without separation of the layers and treated with aqueous 50% CCl_3CO_2H to pH 2 at 4 °C. After centrifugation and dialysis of the supernatant, a LPS preparation was obtained in a yield of 6.9% of dried cell mass.

2.2. Mild acid hydrolysis and O-deacylation

Mild acid degradation of the LPS was performed with aqueous 2% HOAc or 0.1 M NaOAc buffer pH 4.3 for 1–2 h at 100 °C. After centrifugation at 13,000*g*, the supernatant was fractionated by size-exclusion chromatography subsequently on a column (56 × 2.6 cm) of dextran gel Sephadex G-50 Superfine (Amersham Biosciences) using 0.05 M pyridinium acetate pH 4.5 as eluent and a column (80 × 1.6 cm) of fractogel TSK HW-40 (S) (Toyo Pearl) in 0.1% aqueous HOAc; monitoring was performed using a differential refractometer (Knauer).

OS was obtained in yields of 12–13%. The LPS and OS were treated with 12.5% aqueous ammonia at 37 °C for 16 h to afford LPS_{OH} and DOS, respectively. After centrifugation, LPS_{OH} was purified by gel filtration on dextran gel Sephadex G-50 as above; DOS was analysed without purification.

2.3. Monosaccharide analysis

An OS sample (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were conventionally reduced with $NaBH_4$, acetylated with a 1:1 Ac_2O/pyridine mixture, and analysed by GLC on a Maestro (Agilent 7820) system (Interlab, Russia) equipped with an HP-5 column(Agilent) using a temperature program from 160 (1 min) to 290 °C at 7 °C min^{-1}. An OS sample (1 mg) was heated with 2 M HCl (from AcCl) in (*S*)-2-octanol (120 °C, 2 h), 2-octanol was removed with a stream of air, the residue was acetylated and analysed by GLC as above.

2.4. NMR spectroscopy

NMR spectra were recorded at 30 or 60 °C on a Bruker Avance II 600 MHz spectrometer using a 5 mm broadband inverse probehead for solutions in 99.95% D_2O after deuterium exchange by freeze-drying sample solutions in 99.9% D_2O. Sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (internal, δ_H 0, δ_C −1.6) and 85% H_3PO_4 (external, δ_P 0) were used as calibration standards for ^1H, ^{13}C, and ^{31}P NMR chemical shifts, respectively. 2D NMR spectra were obtained using standard Bruker software, and the Bruker TopSpin 2.1 program was used to acquire and process the NMR data. The 2D TOCSY and ROESY spectra were recorded with 60 ms duration of MLEV-17 spin-lock and 150 ms mixing time, respectively. The ^1H,^{13}C and ^1H,^{31}P HMBC spectra were recorded with 60 ms delay for evolution of long-range spin couplings.

2.5. Mass spectrometry

ESI HR MS was performed in the positive or negative mode using a maXis instrument (Bruker Daltonics) [10]. Capillary entrance voltage was set to −4500 or 3200 V, respectively; the drying nitrogen temperature was 180 °C. A sample (~50 ng μL^{-1}) was dissolved in a 1:1 (v/v) H_2O/MeCN mixture and sprayed at a flow rate of 3 $\mu L\,min^{-1}$. Mass range was from *m*/*z* 50–3000 Da, external or internal calibration was done with ESI Tuning Mix (Agilent). The difference between experimental and calculated *m*/*z* values did not exceed 5 ppm. In CID experiments, the activation energy indicated in the figure legends was set to achieve the maximum abundance of fragment peaks. Fragmentation is designated according to the nomenclature [11].

2.6. Analysis of genes

To assign possible gene functions the NCBI BLAST program was used to screen the homologous sequences in the GenBank database.

3. Results and discussion

3.1. Structure elucidation of the O-polysaccharide

3.1.1. Isolation and composition of oligosaccharides

The LPS was isolated from bacterial cells of *E. coli* O84 by the phenol-water extraction and purified by precipitation of proteins and nucleic acids with trichloroacetic acid. The linkage of 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid (Kdo) that connects the carbohydrate and lipid moieties of the LPS is acid-labile, and mild acid degradation is used to cleave it to release the LPS components. However, an attempt to isolate the O-polysaccharide by treatment of the LPS with aqueous 2% acetic acid failed as the polysaccharide chain was depolymerized to afford a mixture of oligosaccharides (OS). Surprisingly, it was also cleaved by degradation under milder conditions, namely sodium acetate buffer pH 4.3, which have been successfully applied for isolation of O-polysaccharides that contain acid-labile ketosidic or glycosyl phosphate linkages (e.g. those of *E. coli* O152 [3] and O172 [2] including the α-D-Glc*p*-1-*P* or α-D-Glc*p*NAc-1-*P* linkage, respectively). Further studies showed that upon LPS degradation, the O84-polysaccharide was cleaved at the β-L-Fuc*p*-1-*P* linkage, which is significantly less stable than the linkages mentioned above, probably due to a different anomeric configuration (β versus α) and/or 6-deoxygenation of the sugar moiety. The OS was found to contain the major phosphorylated pentasaccharide (OS1) that corresponds to the O-unit and the respective minor dephosphorylated pentasaccharide (OS2).

Sugar analysis of the OS by GLC of the alditol acetates derived after full acid hydrolysis (120 °C, 2 h) revealed fucose, 6-deoxytalose (6dTal), glucose, and GlcNAc in the ratios ~2: 0.7: 0.7: 0.4 (detector response). Under milder hydrolysis conditions (100 °C, 1 h) the ratio of Fuc and 6dTal was ~2: 1 but the relative content of Glc and GlcNAc decreased significantly. The L configuration of fucose and the D configuration of the other monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides [12]. The ^1H and ^{13}C NMR spectra of the OS showed signals for multiple O-acetyl groups at δ_H 2.10–2.22 and δ_C 21.3–21.8 (Me). For further structural analysis, the OS was treated with aqueous ammonia to give a mixture (DOS) of the corresponding O-deacetylated oligosaccharides DOS1 and DOS2.