Structural studies on the *Escherichia coli* O101 lipopolysaccharide found in association with F41 and K99 fimbriae

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**Abstract**

In this study it was shown that the O101 lipopolysaccharide isolated from *Escherichia coli* B41 did not contain an O-specific polysaccharide and that its sugar moiety is probably restricted to the core oligosaccharide. It is characterized by the presence of galactose, glucose, N-acetylglucosamine, heptose and 3-deoxy-D-manno-2-octulosonic acid and the fatty acid composition is typical of an Enterobacteriaceae lipopolysaccharide. Methylation analysis indicated terminal non-reducing galactose and glucose and also 1,2-linked glucose which is a substitution pattern typical of an *E. coli* lipopolysaccharide core oligosaccharide. The obtained structural information is sufficient to explain the previously observed interactions between the O101 lipopolysaccharide and the K99 lectin.

**Keywords:** *Escherichia coli*; Fimbriae; Lipopolysaccharide; K99 fimbriae; LPS binding

1. **Introduction**

The lipopolysaccharides (LPS) of Enterobacteriaceae consist of three regions, the O-polysaccharide (O-antigen) composed of oligosaccharide repeating units, the core oligosaccharide and the lipid A region. The core oligosaccharide connects the O-polysaccharide to lipid A which anchors the LPS in the cell envelope.

*Enterotoxigenic* *Escherichia coli* (ETEC) cause diarrhea in both humans and animals. They have two major virulence determinants: toxins and specific colonization factors or adhesins, acting as lectins. For example, F41 and K99 lectins are involved in the adhesion of the bacteria to intestinal epithelial cells of pigs, lambs and calves [1]. It was observed that relatively few O-serotypes are associated with ETEC of veterinary significance [2].

Given that the serotype O101 was found in association with both F41 and K99, either together or alone, and since it was suggested that the O101 O-antigen is an effective protective antigen in an infant mouse model [3], the decision was taken to clone the O101 LPS [4]. However, the chemical structure of the O101 LPS had not yet been investigated despite the fact that knowledge of its structure is essential to understand its possible involvement in the adhesion phenomenon. In this report we describe the isolation and
the partial characterization of the O101 LPS from the reference strain B41. The obtained structural information is sufficient to explain the previously observed interactions between the O101 LPS and the K99 lectin [5,6].

2. Material and methods

2.1. Bacteria, growth conditions and purification of K99 fimbriae

*E. coli* strain B41 (O101; K−; F41; K99) was obtained from the Institut Pasteur Paris (IP 7950). The buffered semi-synthetic minca medium (minca) was supplemented with 1 g of yeast extract per liter [5]. For solid minca 1.5% agar (Difco) was added. The bacteria were grown on solid minca, 18 h at 37°C, harvested by centrifugation (7500 ×g; 20 min) and washed in saline. The fimbriae were extracted by mechanical shearing and purified as previously described [7].

2.2. Extraction and purification of the LPS

The LPS was extracted by the hot phenol-water procedure [8]. Aqueous phases were combined and exhaustively dialyzed against distilled water. A precipitate, which appeared during dialysis, was removed by centrifugation (4000 ×g, 10 min). The opalescent supernatant (crude extract) was concentrated under vacuum and lyophilized. The LPS was also extracted with the Galanos et al. method [9]. The crude extract (250 mg in 15 ml) was treated at 30-35°C with an equal volume of 3% cetyl trimethyl ammonium bromide (CTAB) added dropwise with mild agitation. The mixture was left for 1 h at room temperature and 18 h at 4°C, then warmed (30-35°C) and centrifuged (10 000 ×g; 30 min at 25°C). The supernatant (S_CTAB_) was made 0.1 M with respect to NaCl and then diluted with ethanol (6 vol). Both fractions were recovered by centrifugation (6400 ×g; 30 min) redissolved in water, dialyzed against distilled water and lyophilized. LPS from *E. coli* O111:B4 and *E. coli* J5 were obtained from Sigma Chemical Co. The LPS from *K. pneumoniae* O1:K2 was extracted as described [10].

2.3. Acid hydrolysis of the LPS

LPS (25 mg) in aqueous 2% acetic acid (6 ml) was treated for 90 min at 100°C and the precipitated lipid A was removed from the cooled solution by centrifugation. The 2-keto-3-deoxyoctonate content was estimated with the thiobarbituric acid assay [11]. The pellet was extracted by 10 ml chloroform-methanol (9:1; v/v) and centrifuged. The aqueous phase was discarded, the clear organic phase was filtered on glass wool in a Pasteur pipette and dried under a nitrogen stream.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of LPS

Purified LPS samples were suspended in 10 mM Tris-HCl (pH 6.8) buffer containing 2.5% SDS and subjected to electrophoresis using a 20% polyacrylamide gel. Silver staining was performed by the Tsai and Frasch method [12].

2.5. General methods

Monosaccharides were identified by gas-liquid chromatography (GLC) [13]. Permethylation was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Monosaccharide composition (molar ratios) of LPS O101 and of the fractions obtained from its acid hydrolysate</th>
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<tbody>
<tr>
<td></td>
<td><strong>LPS</strong></td>
</tr>
<tr>
<td>Galactose</td>
<td>1.00^a</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.65</td>
</tr>
<tr>
<td>Heptose</td>
<td>2.27</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>2.05</td>
</tr>
<tr>
<td>3-Deoxy-D-manno-2-octulosonic acid</td>
<td>2.24</td>
</tr>
</tbody>
</table>

^aGalactose was taken as 1.00.
^bN-Acetylglucosamine was taken as 1.00.
carried out as described [14]. Mass spectra (recorded with a Riber 10-10 mass spectrometer using an electron energy of 70 eV and an ionization current of 0.2 mA) were interpreted by comparison to the data of standard compounds [15].

3. Results and discussion

In a typical experiment 250 mg of crude extract was obtained with the phenol-water procedure from 25 g of biomass. RNA was precipitated with CTAB while LPS was recovered in PCTAB. The recovery from the purification step with CTAB was about 70% (from 250 mg of crude extract, 50 mg of SCTAB and 130 mg of PCTAB were obtained). The homogeneity of the purified O101 LPS was shown after SDS-PAGE and silver staining (Fig. 1, lane 4). The O101 LPS migrated as a diffuse blue-gray colored band. This was compared to the migration of LPS from E. coli (serotypes O111 and J5) and K. pneumoniae (serotype O1). Both the smooth-type LPS from E. coli O111:B4 [16] and from K. pneumoniae serotype O1 [17] migrated as diffuse bands of high molecular masses (Fig. 1, lanes 1 and 2). The J5 LPS from E. coli corresponding to a R mutant of the O111:B4 LPS migrated as a low molecular mass compound (lane 3) very close to the O101 LPS from E. coli (lane 4), which could thus correspond to a rough-type LPS. In the light of this result the O101 LPS was extracted using the phenol/chloroform/light petroleum procedure. No difference was seen in the yield and the composition of the LPS.

The sugar part of the LPS is characterized by galactose, glucose, N-acetylglucosamine, heptose and 3-deoxy-D-manno-2-octulosonic acid (Kdo) (Table 1). The fatty acid composition is typical of an Enterobacteriaceae LPS. The O101 LPS contains lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and β-hydroxymyristic acid (3-OH 14:0).

The acid hydrolysate of the purified LPS yielded 56% water soluble sugar containing material and 28% lipid A fraction. After fractionation of the water soluble material on Sephadex G-50 (Fig. 2) three fractions were obtained. Fraction FI (3 mg) and FII (9 mg) had similar compositions (Table 1), characterized by the absence of Kdo which was present only in FIII (2 mg) as shown by the thiobarbituric acid assay and by GLC sugar composition. No material was detected in the void volume of the gel filtration column indicating the absence of high molecular mass compounds in the acidic hydrolysate of the LPS. This result is in agreement with the electrophoresis data and clearly shows that E. coli O101 LPS did not contain an O-specific polysaccharide.

Preliminary information on the structure of the sugar moiety was obtained by permethylation. The compositions of methylated native LPS and fraction FII were compared (Table 2). The important difference between these two fractions was the absence of permethyl Kdo for FII. 3,4,6-Tri-O-methyl glucoside was the major derivative (corresponding to 1,2-
linked glucose) which is a substitution pattern typical of an *E. coli* LPS core oligosaccharide. 2,3,4,6-Tetra-O-methyl glucoside and 2,3,4,6-tetra-O-methyl galactoside correspond to terminal non-reducing monosaccharides already found in the core region of *E. coli* [18]. Two other derivatives (2,3,4,6,7-penta-O-methyl heptoside and 4,5,7,8-tetra-O-methyl Kdo) were identified by comparison of their mass spectra with those of reference compounds [15].

Although the data presented above are not sufficient to establish the exact chemical structure of the sugar moiety of LPS 0101, it is clear that the LPS from the B41 strain contains only the core oligosaccharide. The core oligosaccharides are generally substituted by phosphate [19] which could confer the pronounced acidic nature of the 0101 LPS.

In a previous study [5] we showed that the major problem encountered in the purification of K99 fimbriae was the contamination by outer membrane components, including LPS. In that study the extraction of the fimbriae was carried out with the heat treatment procedure. We found that the precipitate which appeared during biotinylation of the K99 fimbriae via their carboxyl groups (carbodiimide chemistry, pH 4.8) was due to the presence of LPS [5]. The same phenomenon was observed when mixing lysozyme with LPS in equivalent conditions. We concluded that this coprecipitation was due to interactions between the acidic LPS and the K99 fimbriae (or lysozyme), having an isoelectric point between 10 and 11. The purification procedure [5] was improved by the use of mechanical shearing instead of the heat treatment and gave essentially LPS-free fimbriae in the crude extract [7]. No precipitation occurred when the mechanical-shearing purified K99 fimbriae were biotinylated via their carboxyl groups but when the fimbriae were mixed with purified 0101 LPS, a precipitate was formed during the biotinylation reaction.

Recent experiments suggested a possible role for LPS in the formation of K99 fimbriae [6]. In fact, when purified K99 fimbriae were subjected to polyacrylamide gel electrophoresis the K99 major subunit reacted specifically with LPS antiserum. This result suggested [6] that the purified K99 major fimbrial subunit contained covalently bound LPS. In the same study it was shown that K88ab subunits did not react with LPS antiserum and thus K88ab subunits did not contain covalently bound LPS.

During the present study we demonstrated that the 0101 LPS isolated from *E. coli* B41 did not contain an O-specific polysaccharide and that its sugar moiety is probably restricted to the core oligosaccharide. This part of LPS is generally substituted by several phosphate groups which explains why 0101 LPS is acidic. The interactions we previously observed between the acidic 0101 LPS and the K99 lectin [5] are probably not merely due to electrostatic forces since ion pairs contribute little to the stability of a protein's native structure. A hydrophobic effect and hydrogen bonding forces may also contribute to the stabilization of the interaction. It is possible that the heat treatment used for the extraction of the fimbriae favored the formation of covalent bonds between K99 and 0101 LPS. This may be avoided.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Composition (molar ratios) of partially methylated and acetylated methyl glucosides obtained from methylated LPS</th>
</tr>
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<tr>
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<td>LPS</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methyl galactoside</td>
<td>1.09</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methyl glucoside</td>
<td>1.31</td>
</tr>
<tr>
<td>3,4,6-Tri-O-methyl glucoside</td>
<td>3.68</td>
</tr>
<tr>
<td>3,4-Di-O-methyl glucoside</td>
<td>1.00*</td>
</tr>
<tr>
<td>4,6-Di-O-methyl glucoside</td>
<td>0.81</td>
</tr>
<tr>
<td>2,3,4,6,7-Penta-O-methyl heptoside</td>
<td>0.82</td>
</tr>
<tr>
<td>4,5,7,8-Tetra-O-methyl Kdo</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*3,4-Di-O-methyl glucoside was taken as 1.00.
by the use of mechanical shearing for the extraction of the K99 fimbriae [7]. Interestingly Pilipcinec et al. [6] used the mechanical-shearing method for the extraction of the K88ab fimbriae and this could explain why LPS was not found associated with K88ab sub-units. Indeed we did not observe the presence of LPS during the purification of K88ab, K88ac and K88ad fimbriae [20]. Moreover, unlike K99 fimbriae, the pl of K88ab fimbriae is acidic and the interactions with an acidic LPS are less probable.

Acknowledgments

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References