Isolation and characterisation of the lipopolysaccharide from *Xanthomonas hortorum* pv. *vitiens*

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Received 9 July 1999; accepted 23 August 1999

**Abstract**

*Xanthomonas hortorum* pv. *vitiens* is a Gram-negative bacterium that acts as the causative agent of bacterial leaf spot and headrot in lettuce. The lipopolysaccharide (LPS) of this bacterium is suspected to be an important molecule for adhesion to the plants. We have isolated the LPS, prepared the lipid A and the polysaccharide moieties thereof, and characterised all preparations by compositional analysis. Main sugar components are rhamnose and 3-acetamido-3,6-dideoxy-galactose which presumably furnish the O-specific polysaccharide. Other sugars are mannose, glucose, 6-deoxygalactose (fucose), and galacturonic acid, which should be core region constituents, and glucosamine, which builds up the carbohydrate backbone of lipid A. The LPS contains several phosphate groups, most of which are present in the core region. The main fatty acids in the lipid A are C10:0, 3-OH-C10:0 and 3-OH-C12:0. The latter is the only amide-linked fatty acid. Two fatty acids present in small amounts were identified, C8:0 and C11:0. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** *Xanthomonas hortorum*; Lipopolysaccharide; Compositional analysis

**1. Introduction**

We are interested in elucidating the mechanisms of the interaction between plant pathogenic bacteria and their hosts, with special focus on the molecules that play an essential role in this process and, thus, are important virulence factors of the bacteria. Besides extracellular polysaccharides which have been characterised as important virulence factors of plant pathogenic bacteria [1], lipopolysaccharide (LPS) is also thought to affect virulence. However, there are only few investigations of the role of plant pathogenic bacterial LPS, and, moreover, they are contradictory, i.e. with regard to the impact of the O-specific polysaccharide [2–4].

*Xanthomonas hortorum* pv. *vitiens* [5] is a Gram-negative bacterium that acts as the causative agent of bacterial leaf spot and headrot in lettuce. This disease is easily recognised by translucent and water-soaked brown lesions that become dark after a while.
The molecular basis of this disease is not understood. *X. hortorum* is known to produce different phytotoxic metabolites ([6], for other references see here); however, their role in pathogenesis is uncertain. We hypothesise that the LPS of *X. hortorum* pv. *vitians* is important for the interaction between the bacterium and the host, and, thus, represents a prominent virulence factor. To understand the influence of the LPS on bacterial virulence in general and, in particular, on bacteria-plant surface interactions, the determination of the structure of the LPS is a prerequisite. To date, there are only two reports that deal with the sugar composition of LPS from *X. hortorum* [7,8]; however, it is not known to which pathovars these bacteria belonged. Both reports agree in that LPS from *X. hortorum* does not contain heptose, a molecule which is a characteristic constituent of the majority of LPS [9]. In this report, we present the isolation, purification and compositional analysis of LPS from *X. hortorum* pv. *vitians* strain NCPPB 1839. Also, lipid A and the O-specific polysaccharide were prepared and characterised.

2. Materials and methods

2.1. Bacteria and bacterial LPS

*X. hortorum* pv. *vitians* strain NCPPB 1839, obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK (NCPPB), was grown in Watanabe broth for 3 days as described [6] and then lyophilised. Dry cells (4.45 g), obtained from 12 l culture, were suspended in 70 ml of ultrapure Milli-Q water and extracted in the phenol phase of the hot phenol/water extraction [10]. The phenol phase was dialysed (molecular mass cut-off of the dialysis tube: 3500 Da) for 2 days and then lyophilised. This sample was then dissolved in ultrapure Milli-Q water (30 ml) and dialysed as described above. The sample was lyophilised to yield a crude LPS (370 mg, 8% of cell dry mass).

For further purification, the crude LPS fraction (195 mg) was suspended in water and centrifuged (2500×g, 30 min, 20°C; yield of sediment: 20 mg, 10% of crude LPS) and the supernatant was then ultracentrifuged (100000×g, 4 h, 4°C; yield of sediment: 67 mg, 34%). The supernatant (55 ml) was poured into 220 ml of absolute 2-propanol and allowed to precipitate at −18°C for 16 h. After centrifugation (13000×g, 1 h, −20°C, yield of supernatant after dialysis and lyophilisation: 48 mg, 25%), the sediment containing the purified LPS was dialysed, then lyophilised (yield 24 mg, 12% of crude LPS) and used for all investigations.

2.2. Compositional analyses

Analyses of 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo), neutral sugars (as alditol acetates), glucosamine (GlcN), and organic bound phosphate [11] were performed as described. For the determination of uronic acids, 500 mg of LPS was methanolysed (85°C, 45 min) in 0.1 M methanolic HCl, and the sample was dried by rotary evaporation. Then, it was dissolved in water:methanol (4:1, v/v) and sodium borodeuteride was added. Reduction of the carboxyl group was allowed to proceed at 4°C for 16 h. Conversion of the sodium borodeuteride to boric acid was done by adding a few drops of 2 M HCl until bubbling ceased. The sample was then dried by rotary evaporation, followed by three rotary evaporation steps from a solution of 1% acetic acid in methanol, and two from methanol alone. Neutral sugar analysis of the sample was as described [11]. Analyses of ester- and amide-bound fatty acids were performed as described [12].

2.3. Isolation of lipid A and the O-specific polysaccharide

The LPS (13.5 mg) was hydrolysed (100°C, 2 h) in 1% aqueous acetic acid and then centrifuged (2500×g, 30 min), and the sediment (lipid A) and the supernatant (O-specific polysaccharide core fraction) were lyophilised (yields: 2.0 mg lipid A, 15% of purified LPS; 11.3 mg O-specific polysaccharide core
fraction, 84%). The O-specific polysaccharide core fraction was further purified by gel permeation chromatography on a column (50×1.5 cm) of Bio-Gel P10 in 10 mM aqueous NH₄HCO₃.

3. Results and discussion

3.1. Isolation and purification of the LPS

The phenol/water extraction method of lyophilised bacteria of *X. hortorum* pv. *vitiens* strain NCPPB 1839 yielded a crude LPS preparation which also contained other (phospho)lipids and glycans (data not shown) and, thus, was employed in further purification, single steps of which were monitored using fatty acid analysis. Part of the glycolipid material could be separated by centrifugation from a suspension in water, followed by ultracentrifugation. Of this step, the fraction that was present in the supernatant was precipitated in cold 2-propanol, which gave purified LPS, for the purity of which the main criteria were (i) the presence of 3-hydroxy fatty acids and (ii) the absence of typical phospholipid fatty acids, i.e. C16:0 and C16:1. The yield of purified LPS was 12% of crude LPS.

3.2. Compositional analysis of the LPS

The data of monosaccharide, fatty acid and phosphate analyses are listed in Table 1. Sugar constituents are rhamnose (Rha), 3-amino-3,6-dideoxygalactose (3-aminofucose, Fuc3N), fucose (Fuc), mannose (Man), glucose (Glc), glucosamine (GlcN), galacturonic acid (GalA), and Kdo. Thus, the LPS contains common sugars including Fuc3N that has been identified in O-specific polysaccharides of many bacteria, also in strains of plant pathogenic *Pseudomonas syringae* and *P. fluorescens* [13,14]. Interestingly, neither 1-glycero-δ-manno- nor δ-glycero-δ-manno-heptose was identified, which are constituents of the core regions of many bacteria [9]. However, several bacteria synthesise heptose-free LPS, e.g. *Acinetobacter* and *Chlamydia* [9]. A very high content of organic bound phosphate was identified, which is unusual for most LPS investigated so far. However, high numbers of phosphate residues have been identified in LPS of *P. aeruginosa* strains, most of which were attributed to the core region [9]. Main fatty acids are 3OH-C12:0, 3OH-C10:0, and C10:0. This fatty acid composition is similar to that of lipid A of LPS from *P. aeruginosa* [15], and 3OH-C10:0 has been identified in lipid A from some phototrophic bacteria and *Sphaerotilus natans* [15].

3.3. Compositional analysis of lipid A and O-specific polysaccharide

Cleavage of the LPS was possible with 1% aqueous acetic acid, indicating that the O-specific polysaccharide core moiety is linked to the lipid A via a Kdo molecule. Compositional analysis of the lipid A (Table 1) revealed the presence of GlcN and organic bound phosphate in an approximate molar ratio of 2:4, suggesting the presence of two diphasphate groups at a GlcN-disaccharide backbone. Only small amounts of 2-aminoethanol were identified, indicating that minor amounts of these diphasphate groups are further substituted with this compound. Diphasphate or diposphodiester groups present at O1 and O4 of the lipid A backbone have been identified in lipid A of *Neisseria meningitidis*, *Campylobacter jejuni*.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (nmol mg⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>LPS</td>
<td>Lipid A</td>
</tr>
<tr>
<td>GlcN</td>
<td>149</td>
</tr>
<tr>
<td>Kdo</td>
<td>49</td>
</tr>
<tr>
<td>Rha</td>
<td>1906</td>
</tr>
<tr>
<td>Fuc</td>
<td>104</td>
</tr>
<tr>
<td>Fuc3N</td>
<td>613</td>
</tr>
<tr>
<td>Man</td>
<td>99</td>
</tr>
<tr>
<td>GalA</td>
<td>78</td>
</tr>
<tr>
<td>P</td>
<td>1423</td>
</tr>
</tbody>
</table>

*EtN*, 2-aminoethanol.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>total</th>
<th>total</th>
<th>ester-linked</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10:0</td>
<td>31</td>
<td>190</td>
<td>180</td>
</tr>
<tr>
<td>C11:0</td>
<td>–</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>3OH-C10:0</td>
<td>19</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>3OH-C12:0</td>
<td>72</td>
<td>418</td>
<td>150</td>
</tr>
</tbody>
</table>

*tr*, traces.
*n.d., not determined.
*EtN*, 2-aminoethanol.
rhinosurus, Rhodobacter capsulatus, and Moraxella catarrhalis [15], though not in P. aeruginosa. The fatty acids (about five residues per GlcN disaccharide) identified in the LPS analyses are all present in the lipid A moiety. The analysis of the ester-linked fatty acids (Table 1) identified C10:0 (quantitatively), 3-OH-C10:0 (quantitatively), and about one third of the amount of 3OH-C12:0. Thus, 3OH-C12:0 is the only amide-linked fatty acid, as identified also in P. aeruginosa lipid A [15].

The O-specific polysaccharide core fraction was further separated using gel permeation chromatography on Bio-Gel P10. Two major fractions were obtained, eluting with the void volume of the column and shortly after it, respectively. The first contains all sugar components except GlcN and Fuc, whereas in the second only GlcN is missing. Both fractions contained organic bound phosphate, though in relatively low amounts. We assume that apart from those from lipid A all other phosphate residues are linked to the core region. Major sugars in both fractions are Rha and Fuc3N in an approximate molar ratio of 3:1, indicating a repeating unit of the polysaccharide (of strain NCPPB 45) has been characterised [17], consisting of a hexasaccharide repeating unit of 4 Rha, 1 Glc, and 1 GalA residues.

Phytopathogenesis in general is a complex process which may involve a variety of factors, the particular roles of which are important to elucidate. With regard to LPS as a major surface component of Gram-negative plant pathogenic bacteria, its structural characterisation clearly contributes to a deeper understanding of its function in the specific interaction between bacteria and the plant cell surface. We have characterised for the first time the complete composition of LPS from a plant pathogenic X. hortorum strain that induces typical leaf spot and head-rot in lettuce. The results described in this paper represent the basis for further investigations on the structure-function relationship of the LPS from X. hortorum pv. vitians strain NCPPB 1839. Two earlier investigations [7,8] on the LPS from two other strains of X. hortorum mainly dealt with sugar compositions, which is different in the case of strain T646 [7], but shows similarities (Rha and Fuc3N are present) in the LPS from X. hortorum B-1459 that had been obtained from the phenol phase of the phenol/water extraction [8]. Additionally, one O-specific polysaccharide (of strain NCPPB 45) has been characterised [17], consisting of a hexasaccharide repeating unit of 4 Rha, 1 Glc, and 1 GalA residues.

Acknowledgements

We thank A. Müller and R. Engel for expert technical assistance and Prof. A. Zoïna, Dipartimento di Arboricultura, Botanica e Patologia Vegetale, Università di Napoli Federico II, Portici, Italy, for supplying cells of X. hortorum pv. vitians. The work was supported in the framework of the VIGONI program by the Conferenza Permanente dei Rettori delle Università Italiane (to A.M., R.L., A.E. and M.P.) and the Deutscher Akademischer Austauschdienst (to O.H.).

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