

Chemical characterisation of the lipopolysaccharide from *Erwinia carotovora*

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The lipopolysaccharide (LPS) from *Erwinia carotovora*, strain IFO 14082 has been isolated and found to contain D-glucose, L-glycero-D-manno-heptose, 2-acetamido-2-deoxy-D-glucose and 3-deoxy-D-manno-octulosonic acid. The main fatty acid components of lipid-A of the LPS are C12:0, 3-OH-C12:0, C14:0, 3-OH-C14:0 and C16:0. The O-specific side chain (PS), obtained from the LPS, shows the presence of D-glucose and L-glycero-D-manno-heptose in a molar ratio of 7:3. The primary structural features of the O-specific side chain have been established by methylation analysis and Smith degradation together with ^1H and ^{13}C NMR spectral data.

Erwinia carotovora is a pectolytic plant-pathogenic bacterium that causes the soft rot disease of potato and other hosts.^{1,2} It is a Gram-negative organism and contains a lipopolysaccharide (LPS), which is a major amphiphilic glycolipid anchored in the outerspace-leaflets of the outerspacemembrane of the bacteria. The composition and structure of the LPS are important parameters for the chemotaxonomic characterization of microbial species and for the determination of their positions in the phylogenetic tree.³ Plant-associated bacteria are assumed to interact with their hosts where the LPS is reported to be involved in a binding mechanism with the host cells. Ecological and epidemiological studies of these organisms emphasized the need to differentiate among different strains.⁴ The somatic antigen on which carotovora serogroups are defined have not been identified but preliminary evidence suggests them to be LPS.^{5,6} Although compositional analysis of the LPS from *E. carotovora* have already been reported⁷⁻¹¹, the data are not comprehensive. The lipid-A part of the LPS confers stability to the outerspacemembrane, renders it less permeable to lipophilic molecules and stabilizes the conformation of the biologically active membrane proteins. Detailed chemical studies on the LPS from *E. carotovora* is therefore a prerequisite for complete understanding of the basis of serological classification and the role of LPS in bacterial

pathogenicity. We report here the chemical composition of the LPS from *E. carotovora* (strain IFO 14082) and structural features of its O-specific side chain (PS).

Results and Discussion

E. carotovora, IFO 14082 was grown in large quantities on tryptose-tryptone medium and the LPS isolated from dry bacteria by hot phenol-water method in 10% yield. The crude LPS, thus obtained, was purified by ultracentrifugation followed by Cetavlon precipitation and finally by gel-permeation chromatography. Polyacrylamide gel electrophoretic pattern of the purified LPS in presence of sodium dodecyl sulphate is shown in **Figure 1**. Comparison

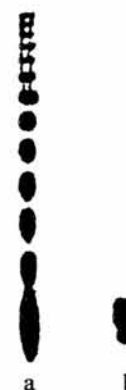


Figure 1 – Electrophoretic migration patterns of LPS (10 μg per lane) obtained by SDS-PAGE. (a) LPS from *S. montevideo* SH 94 S-type, (b) LPS from *E. carotovora*.

of migration patterns of the LPS with that of *S. montevideo* S-type strain revealed the presence of short sugar chains. Treatment of the LPS with mild acid afforded the lipid-A and the PS having $[\alpha]_D +47^\circ$ (*c* 0.5, water).

The chemical composition of LPS, PS and lipid-A from *E. carotovora* are given in **Table I**. Glucose was found to be the major sugar constituent of the LPS together with L-glycero-D-manno-heptose, 2-acetamido-2-deoxy-D-glucose, 3-deoxy-D-manno-2-octulosonic acid (KDO), and traces of D-galactose and L-rhamnose. The LPS also contained phosphate; however, the amount was not determined. Fatty acid analysis of the LPS showed the presence of C12:0, 3-OH-C12:0, C14:0, 3-OH-C14:0 and C16:0. The deacetylated and dephosphorylated PS contained D-glucose and L-glycero-D-manno-heptose in a molar ratio of 7:3 together with traces of L-rhamnose, D-galactose and KDO that are attributed to the presence of some minor contaminants.

The ^1H NMR spectrum of the polysaccharide

Table I — Chemical composition of LPS from *E. carotovora*

Sugars or fatty acids	LPS(%)	O-specific PS(%)	Lipid A(%)
D-Glucose	34.30	53.07	—
LD-Heptose	14.71	22.72	—
D-Glucosamine	4.28	—	15.30
KDO	6.52	Traces	—
L-Rhamnose	Traces	Traces	—
D-Galactose	Traces	Traces	—
C12:O	1.57	—	4.71
3-OH-C12:O	1.31	—	4.0
C14:O	1.26	—	3.65
3-OH-C14:O	13.23	—	38.78
C16:O	3.50	—	9.5

Table II — Data of methylation analysis for *E. carotovora* polysaccharide and its Smith degradation products

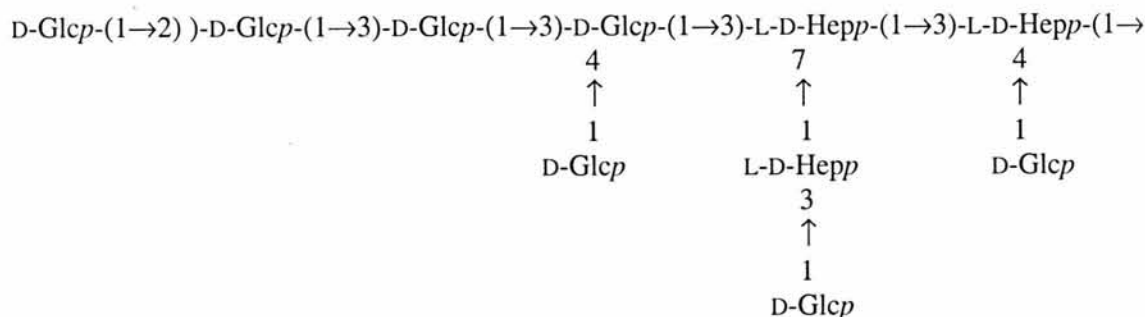
Methylated sugars ^a	Poly-saccharide ^b	Oligo-saccharide I	Oligo-saccharide II
2,3,4,6-Tetra-O-Me-Glcp	4	1	1
2,3,4,6-Tetra-O-Me-Manp	—	1	—
2,4,6,7-Tetra-O-Me-Hepp	1	—	1
2,4,6-Tri-O-Me-Glcp	1	1	—
3,4,6-Tri-O-Me-Glcp	1	—	—
2,4,6-Tri-O-Me-	—	1	1
2,6,7-Tri-O-Me-Hepp	1	—	—
2,4,6-Tri-O-Me-Hepp	1	1	—
2,6-Di-O-Me-Glcp	1	—	—

^a2,3,4,6-Tetra-O-Me-Glcp = 1,5-di-O-Acetyl-2,3,4,6-tetra-O-methylglucitol, etc.

^bvalues are in molar ratios

showed eight broad singlets at δ 5.55, 5.45, 5.35, 5.30, 5.25, 5.20, 5.16, and 4.81 together with two doublets at δ 4.63 ($J = 8$ Hz) and 4.42 ($J = 7.5$ Hz) for ten anomeric protons. These eight broad singlets appearing above δ 4.80 probably indicate α -glucopyranosides and α -heptopyranosides because anomeric protons of β -glucopyranosides do not appear above 4.80 ppm. The two doublets appearing at δ 4.63 and 4.42 must represent two anomeric protons of β -glucopyranosides. The ^{13}C NMR spectrum of the polysaccharide contained signals for anomeric carbons at δ 97.7, 97.8, 97.9, 98.6, 101.3, 101.7, 102.0, 102.8, 103.7 and 104.5 ppm respectively. These values corroborate the results of the proton NMR spectrum. The eight signals below δ 103 probably indicate five α -glucosides and three α -heptosides. The other two signals at δ 103.7 and 104.5 must be due to anomeric carbons of β -glucosides. This takes into account the fact that both monosaccharides having α - and β -manno configurations give C-1 signals at around δ 102.¹²

The results of methylation analysis of PS are given in **Table II**. GC-MS data of the alditol acetates obtained from the methylated PS showed the presence of 2,3,4,6-tetra-O-methylglucose, 3,4,6-tri-O-methylglucose, 2,4,6-tri-O-methylglucose, 2,6-di-O-methylglucose, 2,4,6,7-tetra-O-methylheptose, 2,6,7-tri-O-methylheptose and 2,4,6-tri-O-methylheptose as major constituents in a ratio of 4:1:1:1:1:1. These results indicate the linkages of different sugar moieties and also show that the PS has a branched structure having four non-reducing terminals occupied by glucose residues. Methylation analysis before and after dephosphorylation of O-PS determines the location of phosphate in the heptose residues. Methylated products from phosphorylated heptose were obtained in non-stoichiometric amounts. However, after dephosphorylation the yield of 3,4-linked heptose increased and replaced by 3,4,7-linked heptose. This proved that 3,4-linked heptose was phosphorylated at C-7. The sequence of monosaccharide residues in PS was determined by Smith degradation. Compositional analysis of the degraded product I showed the presence of D-glucose, D-mannose (originating from 3- and 3,4-linked heptoses) and LD-heptose in a molar ratio of 2:2:1. The ^1H NMR spectrum of the Smith degraded product showed anomeric signals as broad singlets at δ 5.70, 5.62, 5.50, 5.38 and 5.31 respectively indicating α -glycosidic nature of the constituent sugars.



III

Methylation analysis of **I** (Table II) showed the presence of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4,6-tetra-*O*-methylmannose, 2,4,6-tri-*O*-methyl-mannose, 2,4,6-tri-*O*-methyl-glucose and 2,4,6-tri-*O*-methyl-heptose in equimolar ratios. These results indicate that the periodate resistant moiety of PS is a branched pentasaccharide in which one glucopyranose and one mannopyranose unit are occupying the nonreducing terminals of the chain containing a 1,3-linked glucopyranose, 1,3,7-linked heptopyranose and a 1,3-linked mannopyranose moieties. The oligosaccharide **I** was subjected to a second periodate oxidation, reduction and mild acid hydrolysis. The resulting product on purification over a column of Bio-Gel P-2 (70 cm $\times$ 1 cm) gave a single product **II**. Compositional analysis of the product showed equimolar amounts of mannose, glucose and heptose. Methylation analysis of **II** showed the presence of a terminal glucose, a 3-linked heptose and a 3-linked mannose (Table II).

The electrophoretic mobility of PS in SDS-PAGE and the results of compositional analysis and NMR data suggest that the *O*-specific side-chain is a nonrepeating deca-saccharide. From these results together with the data obtained from methylation analysis and periodate oxidation studies, the tentative structure **III**, among a few other possibilities, can be assigned to the *O*-specific side-chain. This study also indicates that *E. carotovora* strain IFO 14082 produces rough-form LPS carrying truncated *O*-chains when grown in the laboratory. It is possible that wild type isolates obtained from the field might contain different, e.g. smooth form LPS that are not truncated.

Experimental Section

Bacteria and lipopolysaccharide. *E. carotovora* (strain IFO 14082) used in this study was obtained from the culture collection of The Institution of Fermentation, Osaka (Japan). The bacteria were

grown in large quantities in a laboratory fermenter in tryptose-tryptone medium (pH 6.8) containing yeast extract and glucose as carbon source at 37 °C under aerobic conditions. The cells were harvested at an early stationary phase by gently shaking the cultures with 0.85 % saline and isolated by centrifugation. The bacterial cells were then thoroughly washed with water and freeze-dried. The LPS was isolated from the dry cells (~10 g) using the hot phenol-water method.¹³ The water extract was dialysed and then freeze-dried to yield the crude LPS containing nucleic acid. The latter was removed by precipitation with Cetavlon and the LPS isolated from the supernatant after dialysis and freeze-drying. The LPS thus obtained was further purified by passing through a column of Sephadex G-100 using pyridine-acetic acid buffer (pH 4.5) as eluent. The eluate was collected in 5 mL fractions while monitoring with a Waters Associates Model 403 differential refractometer fitted with a recorder. Appropriate fractions were combined and the buffer was removed by dialysis and lyophilisation to afford ~300 mg of LPS. The LPS contained a trace amount of phosphate which was removed by treatment with aq. HF (48%) at 4 °C for 48 hr. The *O*-specific side chain (PS) was isolated from LPS by mild acid hydrolysis¹⁴ (1.5% acetic acid, 100 °C, 2 hr). The precipitated lipid-A was collected by centrifugation and the supernatant was lyophilised and then passed through a column of Sephadex G-50 using pyridine-acetic acid buffer to afford PS. De-*O*-acetylation of the PS was effected with 0.2 M NaOH at 25 °C for 24 hr. The solution was dialysed, concentrated and lyophilized to yield ~220 mg of pure PS.

Analytical methods. The monosaccharides from LPS or PS were liberated¹⁵ by hydrolysis with 2 M trifluoroacetic acid at 120 °C for 2 hr. The products were then converted into their alditol acetates¹⁶ and analysed by GLC using a Hewlett Packard Model 5890 gas chromatograph equipped with a flame

ionisation detector, HP 3365 series chemstation, and an HP-1 fused silica column (0.32 mm $\times$ 30 m) at 220 °C. Fatty acids from LPS or lipid were liberated by hydrolysing the sample with 2 M HCl in methanol at 100 °C for 18 hr and were analysed by GLC as their methyl esters using a SS column containing 3% SE-52 (1.8 m $\times$ 3 mm) at 164 °C. Partially methylated alditol acetates were characterised by GLC and GC-MS on a Fisons Instrument GC fitted with a mass detector (MD 8000 series) on an HP-5 fused silica column (0.32 mm $\times$ 30 m) at 160 °C for 2 min. followed by an increase of 2 °C per min. to 280 °C. 3-D-coxy-D-manno-2-octulosonic acid (KDO) was estimated by the thiobarbituric acid method¹⁷ after hydrolysing the sample with 1 M HCl at 100 °C for 30 min. Optical rotations were measured on a Perkin-Elmer Model 241 MC spectropolarimeter. Polyacrylamide gel electro-phoresis (PAGE) was carried out using trisglycine buffer, (pH 8.4), containing 0.25 % sodium dodecylsulfate (SDS) with 13 % acrylamide running gel and 4 % stacking gel.¹⁸ After pre-electrophoresis at 25 mA, the samples were separated at 18 mA until the dye reached the bottom of the gel. LPS bands were detected after HIO₄ oxidation, with silver nitrate staining of Tsai and Frasch.¹⁹ ¹H and ¹³C NMR spectra were recorded on a Bruker AMX-500 MHz spectrometer in D₂O using acetone as internal standard.

Methylation analysis. Samples (2-4 mg) were methylated according to the method of Ciucanu and Kerek²⁰ and the products were isolated by partition between dichloromethane and water. The products were further purified by passing through a Sep-Pak C18 cartridge.²¹ Permethylated samples were hydrolyzed with 2 M TFA at 120 °C for 2 hr, reduced with NaBD₄, acetylated with Ac₂O in pyridine, and analyzed by GLC or GLC-MS or both.

Periodate oxidation and Smith degradation. The polysaccharide (48 mg) in 0.1 M sodium metaperiodate solution (10 mL) was kept in the dark for 3 days at 24 °C. The excess of periodate was destroyed by the addition of ethylene glycol (0.25 mL) and the resulting polyaldehyde reduced with NaBH₄ (200 mg) at 24 °C for 16 hr. Excess borohydride was destroyed with 50% acetic acid, the solution dialyzed against distilled water for 2 days and the polyol recovered by lyophilization. The polyol was treated with 0.5 M CF₃COOH (10 mL) at 24 °C for 24 hr and the material

fractionated on a column of Bio Gel P-2 (200-400 mesh). The resulting product **I** was subjected to a second periodate oxidation using the same conditions as above except that the sample was kept for only 16 hr in the dark at 24 °C. The Smith type hydrolysis and fractionation of the product was carried out as above to give a single product **II**.

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References

- 1 Dye D W, *New Zealand J Sc*, **12**, **1969**, 81.
- 2 Lund B M, In: *Plant Pathogens*, edited by D W Lovelock, (Academic Press, London) **1979**, p.14.
- 3 Mayer H, In: *The cell membrane*, edited by E Haber, (Plenum Press, New York), **1984**, p. 71.
- 4 Starr M P & Chatterjee A K, *Ann Rev Microbiol*, **26**, **1972**, 389.
- 5 Deboer S H, Copeman R J & Vrugink H, *Phytopathol*, **69**, **1979**, 316.
- 6 Murray J, Fixter L M, Hamilton I D, Perombelon M C M, Quinn C E & Graham D C, *J Appl Bacteriol*, **68**, **1990**, 316.
- 7 De Boer S H, Bradshaw-Rouse J J, Sequeria L & Mcnaughton M E, *Can J Microbiol*, **31**, **1985**, 583.
- 8 Ray T C, Smith A R W, Wait R & Hignett R C, *Eur J Biochem*, **170**, **1987**, 357.
- 9 Sandulache R & Prehm R, *J Bacteriol*, **161**, **1985**, 1226.
- 10 Fukuoka S, Kamishima H, Sode K & Karube I, *Eur J Biochem*, **68**, **1952**, 320.
- 11 Fukuoka S, Knirel A Y, Lindner B, Moll H, Seydel U & Zahringer U, *Eur J Biochem*, **250**, **1997**, 55.
- 12 Agrawal P K, *Phytochemistry*, **31**, **1992**, 3307.
- 13 Westphal O, Luderitz O & Bister F, *Z Naturforsch*, **7b**, **1952**, 148.
- 14 Das S, Ramm R, Kochanowski H & Basu S, *J Bacteriol*, **176**, **1994**, 6550.
- 15 Albersheim P, Nevins D J, English P D & Karr A, *Carbohydr Res*, **5**, **1967**, 340.

- 16 Blackeney A B, Harris P J, Henry R J & Stone B A, *Carbohydr Res*, **113**, **1967**, 91.
- 17 Brade H, Galanos C & Luderitz O, *Eur J Biochem*, **131**, **1983**, 195.
- 18 Basu S, Radziejewska-Labrecht J & Mayer H, *Arch Microbiol*, **144**, **1986**, 213.
- 19 Tsai C M & Frasch C E, *Anal Biochem*, **119**, **1982**, 115.
- 20 Ciucanu I & Kerek F, *Carbohydr Res*, **131**, **1984**, 209.
- 21 Mort A J, Parkar S & Kuo M, *Anal Biochem*, **133**, **1983**, 380.