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STRUCTURAL STUDIES OF THE O-ANTIGEN OF THE LIPOPOLYSACCHARIDE FROM AN AVIRULENT STRAIN (M4S) OF *Pseudomonas solanacearum*

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ABSTRACT

The structure of the O-antigen of the lipopolysaccharide from an avirulent strain (M4S) of *Pseudomonas solanacearum* has been investigated by methylation analysis, n.m.r. spectroscopy, and *N*-deacetylation–deamination, followed by analysis and controlled Smith-degradation of the product. These studies demonstrate that the O-antigen is composed of a tetrasaccharide repeating-unit having the following structure:

→3)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→.

INTRODUCTION

Pseudomonas solanacearum is one of the most destructive bacterial plant-pathogens in the warm regions of the world[1] and is pathogenic for many different species of plants over 33 genera. Qualitative sugar analysis of its lipopolysaccharide (LPS) has been reported[2], but the structure of the LPS is unknown. We now report structural studies of the O-antigen of the LPS of an avirulent strain (M4S) of *P. solanacearum*.

RESULTS AND DISCUSSION

The LPS was isolated from *P. solanacearum* M4S by extraction of cells with phenol–water followed by the removal of nucleic acids by precipitation with Cetavlon[3]. Mild, acid degradation (aqueous 1% acetic acid, 100°, 1.5 h) of the LPS followed by fractionation of the products on Sephadex G-50 gave polysaccharide (PS) and oligosaccharide fractions. The latter were not investigated further.

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TABLE I

PERTINENT ^{13}C-N M R CHEMICAL SHIFTS (p.p.m.) FOR THE PS (A), TETRASACCHARIDE **3** (B), AND DISACCHARIDE **5** (C)

Sugar unit	*Carbon atom*	*A*	*B*	*C*
→2)-α-L-Rha*p*-(1→	C-1	101.6 ($^1J_{C,H}$ 173 Hz)	101.7	
	C-2	77.1[a], 78.4[a]	78.1[b]	
	*C*H$_3$	17.6	17.6	
→3)-α-L-Rha*p*-(1→	C-1	100.3 ($^1J_{C,H}$ 170 Hz)	100.3	
	C-3	79.2[a]	79.1[b]	
	*C*H$_3$	17.4	17.6	
α-L-Rha*p*-(1→	C-1		103.2	100.7
	*C*H$_3$		17.6	17.6
→3)-α-D-Glc*p*NAc-(1→	C-1	97.1 ($^1J_{C,H}$ 173 Hz)		
	C-2	54.0		
	C-3	80.1[a]		
	C-6	61.4		
	*C*OCH$_3$	174.6		
	CO*C*H$_3$	22.9		
→3)-2,5-Anhydro-D-mannitol	C-1		62.5[c]	62.5[d]
	C-2		84.0[e]	84.1
	C-3		84.2[e]	84.1
	C-5		82.8	82.7
	C-6		61.9[c]	61.9[d]

[a–e] May be interchanged.

The PS, $[\alpha]_D^{23}$ +22.3° (water), appeared to be homogeneous on the basis of zone electrophoresis and gel-filtration, and its molecular weight was calculated to be ~16,000. The PS contained neutral sugars (mainly L-rhamnose) and 2-acetamido-2-deoxy-D-glucose in the molar ratio ~3:1, with small proportions of D-xylose and D-glucose residues, which, together, accounted for ~84% of the PS.

The 500-MHz, ^{1}H-n.m.r. spectrum of the PS contained, *inter alia*, resonances at δ 1.25 (3 H, *J* 5.5 Hz, Me of L-Rha), 1.31 (6 H, *J* 6.3 Hz, Me of L-Rha), 2.07 (3 H, NAc), 4.89 (1 H, $J_{1,2}$ <1 Hz, H-1 of L-Rha), 4.98 (1 H, $J_{1,2}$ 3.3 Hz, H-1 of D-GlcNAc), 5.02 (1 H, $J_{1,2}$ <1 Hz, H-1 of L-Rha), and 5.19 (1 H, $J_{1,2}$ <1 Hz, H-1 of L-Rha). The ^{13}C-n.m.r. spectrum of the PS (see Table I) contained three signals for anomeric carbons at 101.6, 100.3, and 97.1 p.p.m. with $^1J_{C,H}$ values of 173, 170, and 173 Hz, respectively, indicating that the anomeric configurations of the component sugars are all α.

O-Specific side-chains of LPS are generally composed of oligosaccharide repeating-units, and the results presented so far suggested that the O-antigen of *P. solanacearum* M4S is composed of a tetrasaccharide repeating-unit containing three α-linked L-rhamnosyl and one α-linked 2-acetamido-2-deoxy-D-glucosyl residues.

Methylation analyses of the PS and *N*-deacetylated PS gave the sugar deriva-

TABLE II

METHYLATION-ANALYSIS DATA FOR THE ORIGINAL AND MODIFIED PS FROM *P. solanacearum M4S*

Methylated sugar[a]	T[b]	*Relative mol%*[c]			
		A	B	C	D
1,4,6-2,5-Anhydro-Man[d]	0.64			17.7	52.8
2,3,4-Xyl	0.80	1.6	4.4		
2,3,4-Rha	0.82	3.3	9.0	28.1	47.2
3,4-Rha	0.94	49.0	40.1	30.9	
2,4-Rha	0.96	36.4	40.8	23.3	
2,3,4,6-Glc	1.00	1.6	+		
2-Rha	1.03	5.6	4.8		
4-Rha	1.05	2.4	0.8		

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Retention times for the corresponding alditol acetates relative to that of 2,3,4,6-Glc on column *A*. [c]*A*, original PS; *B*, *N*-deacetylated PS; *C*, tetrasaccharide **3**-(*1-d*); *D*, disaccharide **5**. [d]Parts of this volatile ether and its derivatives were probably lost during concentrations.

tives listed in Table II, columns *A* and *B*, respectively; they were analysed by g.l.c.–m.s. of their partially methylated alditol acetates. 3,4-Di-*O*-methyl- and 2,4-di-*O*-methyl-L-rhamnose were detected as the main products. The small proportions of xylose, glucose, and mono-*O*-methylated rhamnose derivatives were thought to be derived from the contaminating core-oligosaccharides. In addition, 2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose was found and identified by g.l.c.–m.s. of its alditol acetates[4,5], but the analysis of this sugar was qualitative only. The results support the suggestion of a tetrasaccharide repeating-unit, which contains two 2-linked L-rhamnosyl, one 3-linked L-rhamnosyl, and one 3-linked 2-acetamido-2-deoxy-D-glucosyl residues. The lower yield of 2-linked L-rhamnosyl residue than expected suggests the presence of the sequence **1** in the PS, because the glycosidic linkage between hexosamine and rhamnose is not significantly hydrolysed. Thus, the PS is thought to have a repeating unit **2**.

→3)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→

1

→3)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2 or 3)-α-L-Rha*p*-(1→3 or 2)-α-L-Rha*p*-(1→

2

The PS was *N*-deacetylated by treatment with sodium hydroxide–sodium thiophenolate in aqueous methyl sulfoxide. The *N*-deacetylated PS, $[\alpha]_D^{23}$ +1.1° (water), did not contain *N*-acetyl groups, as evident from its ^1H- and ^{13}C-n.m.r. spectra. Deamination of the *N*-deacetylated PS with sodium nitrite in aqueous acetic acid yielded a tetrasaccharide, which was reduced with sodium borohydride

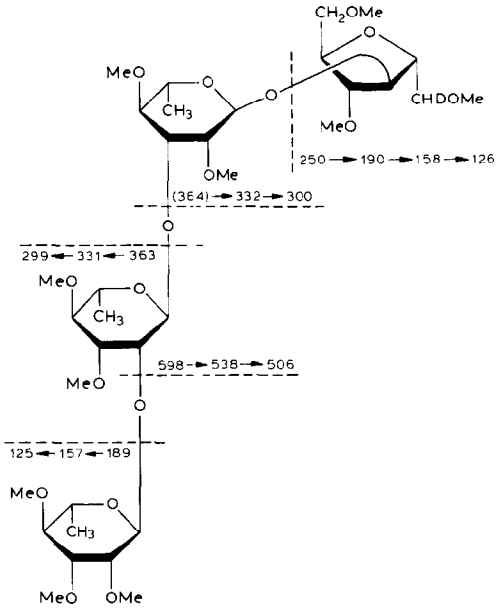
(oligosaccharide for methylation analysis was prepared by reduction using sodium borodeuteride) to give **3** [or **3**-(*1-d* derivative)], which had $[\alpha]_D^{23}$ −55.9° (water).

α-L-Rha*p*-(1→2 or 3)-α-L-Rha*p*-(1→3 or 2)-α-L-Rha*p*-(1→3)-2,5-anhydro-D-mannitol
3

Sugar analysis of **3** revealed L-rhamnose and 2,5-anhydro-D-mannitol in the molar ratio ~3:1. Secondary ion mass spectrometry (s.i.m.s.) of **3**-(*1-d*) gave ions at *m/z* 626 and 627, corresponding to $[M + {}^{23}Na]^+$ and $[M + {}^{23}Na + 1]^+$, respectively. The ^{13}C-n.m.r. data (see Table I) were consistent with the structure **3**.

Methylation analysis of **3**-(*1-d*) (Table II, column *C*) also supported the structure, but gave no information on the sequence of the two L-rhamnosyl residues linked through O-2 and O-3. The identification of 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* by g.l.c.–m.s.[6] confirmed that the 2-acetamido-2-deoxy-D-glucosyl residue was 3-linked in the PS.

G.l.c. of the methylated **3**-(*1-d*) gave one peak (*T* 2.01; *T* 1.00 for methylated cellobiitol on column *B*). G.l.c.–m.s. (see **4**) gave ions of the aA series [*m/z* 189 (100%), 157 (39), and 125 (16)], the baA series [363 (5), 331 (11), and 299 (8)], the dA series [190 (22), 158 (13), and 126 (9)], the cdA series [332 (4) and 300 (2); cdA_1 ion (*m/z* 364) was not observed], the bcdA series [538 (10) and 506 (4)], and the J series [598 ($abcdJ_1$, 2) and 250 (cdJ_1, 2)]. The cbaA-series ions were not observed. The presence of the $abcdJ_1$ ion (*m/z* 598) also indicated the tetrasaccharide nature of **3**.



4

As already mentioned, **3** contained 2- and 3-linked L-rhamnosyl residues. Kärkkäinen[7] and Moor and Waight[8] have reported that 2- and 3-linked glycosyl residues show differences in the A series of fragmentation. Their studies showed that $baA_1<baA_2$ if the non-reducing terminal sugar is glycosidically linked to O-2 of the penultimate sugar, and that $baA_2<baA_1$ if, on the other hand, the terminal sugar is linked to O-3 of the penultimate sugar. In the mass spectrum of methylated **3**-(*1-d*), the intensity of the baA_1 ion (*m/z* 363, 5%) is less than that of the baA_2 ion (*m/z* 331, 11%), suggesting that the non-reducing terminal L-rhamnosyl group is glycosidically linked to O-2 of the next L-rhamnosyl residue. It has also been reported[9,10] that the D series of fragments does not produce so intense an abcJ fragment when the sugar residues a and b are (1→3)-linked as that obtained when the sugar residue b carries a methoxyl substituent on C-3. In the mass spectrum of methylated **3**-(*1-d*), two J-series ions were observed at *m/z* 598 ($abcdJ_1$, 2%) and 250 (cdJ_1, 2%). The absence of the $bcdJ_1$ ion (*m/z* 424) suggested that the second L-rhamnosyl residue from the non-reducing terminal is linked to O-3 of the third L-rhamnosyl residue. Thus, from the g.l.c.–m.s. studies of the methylated **3**-(*1-d*), the sequence of the linkage of the L-rhamnosyl residues in **3** is suggested to be 1→2, 1→3.

To determine the linkage sequence of the L-rhamnosyl residues, **3** was treated with sodium metaperiodate, and the product was reduced with sodium borohydride and hydrolysed under mild, acid conditions (50mM sulfuric acid, room temperature, 24 h). Then the product was fractionated on Sephadex G-15 to give **5**, which was eluted in the disaccharide region. The ^{13}C-n.m.r. spectrum (see Table I) was consistent with the structure **5**. Methylation analysis (Table II, column *D*) revealed terminal L-rhamnosyl groups and 3-linked 2,5-anhydro-D-mannitol residues in almost equal amounts. G.l.c. of methylated **5** gave one peak (*T* 0.78), and g.l.c.–m.s. gave ions at *m/z* 189 (aA_1 and bA_1), 157 (aA_2), 125 (aA_3), and 249 (abJ_1). The presence of the abJ_1 ion confirmed the disaccharide nature of **5**, and the results support the structure assigned.

α-L-Rha*p*-(1→3)-2,5-anhydro-D-mannitol
5

The production of disaccharide **5** from the tetrasaccharide **3** by controlled Smith-degradation indicates that the linkage sequence of the L-rhamnosyl residues in **3** is 1→2,1→3, consistent with the sequence obtained from g.l.c.–m.s. of methylated **3**-(*1-d*). Thus, the structure of the tetrasaccharide **3** and the PS are determined as **6** and **7**, respectively. This structure is very similar to that of the O-antigen of *Shigella flexneri*[6], except for the anomeric configuration of the 2-acetamido-2-deoxy-D-glucosyl residue, which is β in the *Sh. flexneri* polymer.

α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-2,5-anhydro-D-mannitol
6

→3)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→
7

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at <45° (bath). Optical rotations were measured at 23° with a Jasco DIP-181 polarimeter, using a 100-mm micro-cell. Zone electrophoresis was performed on Whatman GF/A, glass-microfibre paper (47 × 5 cm) with 0.1M sodium tetraborate buffer (pH 9.3) at 1500 V for 30 min, and detection with 20% sulfuric acid in methanol. High-performance, gel-permeation chromatography was conducted with a Waters Solvent Delivery System 6000 constant-flow pump, a Waters R-401 differential refractometer, and a column of Toyo Soda TSK-Gel G4000SW, which was calibrated with standard dextrans, using 0.1M sodium chloride as eluant. G.l.c. was performed with a Shimadzu GC-7A instrument fitted with a flame-ionisation detector and *A*, a glass-capillary column (50 m × 0.28 mm) coated with 3% of OV-101; *B*, a glass column (1 m × 0.32 cm) packed with Chromosorb W coated with 5% of OV-101; and *C*, a Hewlett–Packard fused-silica capillary column (25 m × 0.31 mm) containing cross-linked methyl silicone. Total sugar was determined by the phenol–sulfuric acid method[11]. Hexosamine were determined after hydrolysis of the sample in 4M hydrochloric acid at 110° for 4 h. After removal of the acid, the hydrolysate was passed through a column of Dowex 50 (H^+) resin, and the hexosamine, which was eluted with 2M hydrochloric acid, was determined colorimetrically[12]

Neutral sugar analysis was performed by g.l.c. (column *A*) of the alditol acetates[13]. Methylation and g.l.c.–m.s. (column *C*) analyses were performed as described previously[14]. The methylated oligosaccharide-alditol was recovered from the reaction mixture by using a Sep-Pak C_{18} cartridge[15] (Waters). G.l.c. and g.l.c.–m.s. of the methylated oligosaccharide-alditol were performed with column *B* (from 150° to 320° at 5°/min) and column *C* (300° isothermal), respectively. Mass spectra were obtained with a Hitachi M-80 (70 eV) and data processor M-003.

S.i.m.s. was obtained using $[Xe]^+$ as primary ions; ~0.5 μL of glycerine matrix was added to the sample on the silver substrate. The acceleration voltages of the primary and secondary ions were 8 and 3 kV, respectively.

N.m.r. spectra for solutions in D_2O were recorded with a Hitachi R90H (^1H, 90.01 MHz; ^{13}C, 22.4 MHz) or Bruker AM-500 (^1H, 500.13 MHz) spectrometer at 30°, using a 5-mm tube, and internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (0.015 p.p.m. from Me_4Si; for ^1H) or internal methanol (49.9 p.p.m. from Me_4Si; for ^{13}C) as the standard. Chemical shifts were expressed in p.p.m. downfield from the standard, and $^1J_{C,H}$ values were determined by the gated, ^1H-decoupler sequence to retain the n.O.e.

Bacteria. — An avirulent strain of *P. solanacearum,* M4S, is a mutant from U-7, a strain highly pathogenic to tobaco and tomato[16].

Preparation of PS. — Bacterial cells (116 g, fresh wt.) were extracted with phenol–water, and the nucleic acid was removed from the extract by Cetavlon[3] to give LPS (5.5 g, dry wt.). LPS (2 g) was treated with aqueous 1% acetic acid (300 mL) for 1.5 h at 100°, the mixture was centrifuged to remove lipid A, and the material in the supernatant solution was purified by gel filtration on a column (85 × 2.6 cm) of Sephadex G-50 to give PS (880 mg), $[\alpha]_D^{23}$ +22.3° (*c* 0.5, water).

N-*Deacetylation*[17]. — A solution of PS (110 mg), sodium hydroxide (1 g), and thiophenol (220 μL) in water (2.2 mL) and methyl sulfoxide (21 mL) was kept at 90° for 16 h, neutralised with M hydrochloric acid, filtered, desalted with Sephadex G-25, and freeze-dried to give *N*-deacetylated PS (70 mg), $[\alpha]_D^{23}$ +1.1° (*c* 0.2, water).

Deamination. — *N*-Deacetylated PS (34 mg) was dissolved in water (1.3 mL), aqueous 33% acetic acid (2 mL) and aqueous 5% sodium nitrite (2 mL) were added, and the solution was kept at 25° for 1 h and passed through a column of Dowex 50 (H^+) resin. A solution of the product in water (2 mL) was treated with sodium borohydride (20 mg) or sodium borodeuteride (20 mg), neutralised with Dowex 50 (H^+) resin, filtered, and concentrated. Boric acid was removed from the residue by codistillation with methanol (5 × 5 mL). Fractionation of this product on a column (85 × 1.6 cm) of Sephadex G-15 yielded oligosaccharide **3** (9 mg), $[\alpha]_D^{23}$ −55.9° (*c* 0.25, water); lit.[6] $[\alpha]_{578}^{25}$ −60°; which was eluted in the tri- to tetra-saccharide region. The mass spectrum of fully methylated **3**-(*1-d*) contained, *inter alia,* the following fragments (relative intensities in brackets): *m/z* 45 (41%), 46 (10), 59 (28), 69 (6), 71 (13), 72 (25), 73 (10), 75 (34), 83 (7), 85 (12), 88 (88), 89 (13), 97 (15), 98 (6), 99 (27), 101 (40), 111 (6), 113 (7), 115 (8), 125 (16), 126 (9), 127 (6), 129 (31), 145 (10), 157 (39), 158 (13), 159 (7), 171 (12), 189 (100), 190 (22), 203 (11), 207 (13), 224 (36), 225 (6), 250 (2), 256 (15), 267 (6), 281 (9), 299 (8), 300 (2), 320 (11), 331 (11), 332 (4), 363 (5), 506 (4), 538 (10), and 598 (2). Data for the methylation analysis of **3**-(*1-d*) are given in Table II, column *C*. The mass spectrum of 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* showed, *inter alia,* peaks at *m/z* 41 (6%), 43 (54), 45 (36), 46 (23), 56 (5), 69 (6), 71 (13), 75 (7), 86 (6), 87 (12), 97 (9), 101 (8), 103 (9), 111 (11), 112 (20), 116 (6), 126 (14), 129 (100), 130 (9), 143 (12), 144 (19), 157 (8), 203 (30), and 204 (5).

Controlled Smith-degradation[18]. — Oligosacharide **3** (4 mg) was dissolved in 0.1M sodium acetate buffer (pH 4.0, 4 mL), and 0.2M sodium metaperiodate (1 mL) was added. The reaction mixture was kept in the dark at 4° for 96 h, excess of periodate was then reduced by adding ethylene glycol (0.2 mL), and the mixture was concentrated and purified on a column of Sephadex G-15. The main fraction (eluted in the di- to tri-saccharide region) was treated with sodium borohydride (40 mg), neutralised with Dowex 50 (H^+) resin, filtered, and concentrated. Boric acid was removed as described above. A solution of the product in 0.05M sulfuric acid (3 mL) was left at room temperature for 24 h, neutralised with barium carbonate, filtered, deionised by treating with Dowex 50 (H^+) resin, concentrated, and purified by gel filtration on Sephadex G-15 to give oligosaccharide **5** (1 mg), which was

eluted in the disaccharide region. The mass spectrum of fully methylated **5** showed, *inter alia,* the following pertinent fragments (relative intensities in brackets): *m/z* 45 (25%), 59 (12), 71 (5), 72 (33), 75 (12), 88 (100), 89 (9), 99 (5), 101 (23), 125 (10), 129 (6), 131 (5), 157 (38), 189 (36), and 249 (26).

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