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Note

The structure of O-specific polysaccharide from *Pseudomonas solanacearum* ICMP 4157*

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Pseudomonas solanacearum is a phytopathogenic, gram-negative microorganism having many heterogeneous biological and biochemical properties[1-3]. The structure of the O-antigen of the virulent strain *P. solanacearum* U-7 and its avirulent mutant M4S has been determined[4,5] as that of a linear polysaccharide having a tetrasaccharide repeating-unit built up of one α-D-Glc*p*NAc and three α-L-Rha*p* residues. The same structure was established[6] for O-specific polysaccharides from *P. solanacearum* strains PDDCC 7859, ICMP 8110, and ICMP 8202, whereas strain ICMP 5712 was shown[6] to produce an O-antigen having a branched pentasaccharide repeating unit with a backbone consisting of one β-D-Glc*p*NAc and three α-L-Rha*p* residues, and a β-D-Xyl*p* group attached as a side chain to one of the α-L-Rha*p* residues. We report, herein, the structure of the O-specific polysaccharide of *P. solanacearum* ICMP 4157.

The lipopolysaccharide of this strain was isolated from bacterial cells by the method of Westphal and Jann[7] and cleaved with dilute acetic acid to give an O-specific polysaccharide, isolated by gel-permeation chromatography on Sephadex G-50.

Acid hydrolysis of the polysaccharide gave arabinose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose which were identified by g.l.c. as alditol acetates. The D configuration of arabinose was determined by g.l.c. of the corresponding (*S*)-2-octyl glycoside according to the method of Leontein *et al.*[8] The D configuration of the amino sugars was established by calculation of the specific optical rotation according to Klyne's rule (see below).

In the ^{1}H-n.m.r. spectrum of the polysaccharide (Table I), signals for three anomeric protons were present at δ 4.91 (d, $J_{1,2}$ 4 Hz), 5.03 (d, $J_{1,2}$ 4 Hz), and 5.08 (m, $J_{1,2}$ $<$4 Hz), for two NAc groups at δ 2.05 and 2.06, and for other protons in the region δ 3.5–4.3. The ^{13}C-n.m.r. spectrum (Table II) also showed the presence of a polysaccha-

* Dedicated to Professor Serge David on the occasion of his 70th birthday.

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TABLE I

^{1}H-N.m.r. chemical shifts (δ) for the O-specific polysaccharide[a]

H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
2-Acetamido-2-deoxy-α-D-glucopyranose (Unit A)					
4.91	3.94	3.94	3.67	3.94	3.66, 3.97
2-Acetamido-2-deoxy-α-D-galactopyranose (Unit B)					
5.03	4.20	4.02	4.00	4.26	3.69 (2H)
β-D-Arabinofuranose (Unit C)					
5.08	4.12	4.15	3.88	3.73 (2H)	

[a] Coupling constants J were not determined (exept for $J_{1,2}$ 4 Hz for units B and C) owing to multiple coincidences of the resonances for vicinal protons. Additional signals: NAc at δ 2.05 and 2.06 (both s.).

TABLE II

^{13}C-N.m.r. chemical shifts (δ)[a]

Unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
O-Specific polysaccharide						
→6)-α-D-Glc*p*NAc-(1→ (A)	99.3	55.1	73.2	79.7	70.1	67.3
4 ↑						
→4)-α-D-Gal*p*NAc-(1→ (B)	99.4	51.6	68.4	78.5	71.7	61.6
β-D-Ara*f*-(1→ (C)	103.0	77.6	74.2	82.8	62.2	
Smith-degraded polysaccharide[b]						
→6)-α-D-Glc*p*NAc-(1→ (A)	99.2	54.9	72.1	70.9	71.4	66.5
	(98.9)	(55.3)	(72.0)	(71.4)	(71.5)	(66.9)
-4)-α-D-Gal*p*NAc-(1→ (B)	98.1	51.0	68.3	78.3	72.5	61.2
	(97.8)	(51.0)	(68.5)	(77.9)	(72.4)	(61.7)

[a] Additional signals: NAc at δ 23.1–23.4 (CH_3), 175.7–176.0 (CO). [b] Values calculated by the method of Lipkind *et al.*[10] are given in parentheses.

ride having a trisaccharide repeating unit (signals for three anomeric carbon atoms at δ 99.3, 99.4, and 103.0), two of the components being acetamidodeoxy sugars [signals for two carbon atoms bearing a nitrogen atom at δ 51.6 and 55.1; and for two NAc groups at δ 23.1 and 23.4 (Me), and 175.7 and 175.9 (CO)]. The total number (17) of signals for sugar carbon atoms in the ^{13}C-n.m.r. spectrum indicated that two of the constituent monosaccharides were hexose derivatives, and one was a pentose derivative. Therefore, the O-specific polysaccharide has a trisaccharide repeating unit consisting of residues of Ara, GlcNAc, and GalNAc.

The ^{1}H-n.m.r. spectrum of the polysaccharide was assigned with the help of 2D-homonuclear, shift-correlated spectroscopy in combination with single-relayed, coherence transfer spectroscopy (Table I). Uncertainty in several assignments, introduced by the coincidence of the proton resonances, was resolved by application of

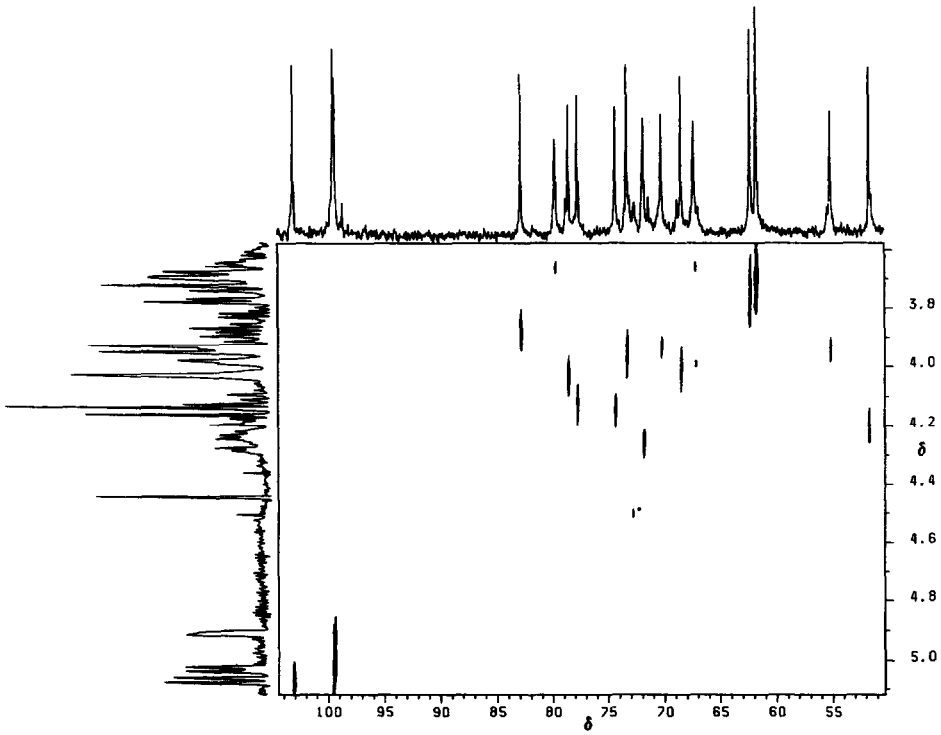


Fig. 1. 2D Heteronuclear ^{13}C–^{1}H shift-correlated spectrum of O-specific polysaccharide of *P. solanacearum* ICMP 4157 (regions of F_2, δ 110–45; and F_1, δ 3–5.3). The respective 1D ^{13}C- and ^{1}H-n.m.r. spectra are displayed along the F_2 and F_1 axes, respectively. The signals for the NAc groups, folded within the spectral window, are marked with an asterisk.

heteronuclear ^{13}C–^{1}H shift-correlated spectroscopy (Fig. 1). This also allowed us to assign the signals in the ^{13}C-n.m.r. spectrum of the polymer (Table II).

The monosaccharide residues were arbitrarily designated as units A, B, and C in order of increasing chemical shift values for the corresponding anomeric protons and carbon atoms. The positions at δ 55.1 and 51.6 of the signals for C-2 of units A and B indicated[9,10] that these units were the Glc*p*NAc and Gal*p*NAc residues, respectively, and, hence, unit C was the Ara residue. The $J_{1,2}$ value of $\leqslant$4 Hz indicated that the residues of both amino sugars have the α-D configuration. The ^{13}C-chemical shifts for unit C (Table II) proved[9] that the Ara unit has a β-D-furanose structure and is unsubstituted. As judged by the relatively low-field positions at δ 79.7 and 78.5 of the signals for C-4, units A and B are 4-substituted. Analogously, a relatively low-field position at δ 67.3 of the signal for C-6 of one of the HexNAc residues was indicative of 6-substitution of unit A or B.

On irradiation of the H-1 of unit A at δ 4.91, together with n.O.e.'s on H-2 and, due to spin diffusion, H-3,4 of the same unit at δ 3.94 and 3.67, a significant n.O.e. was observed for H-4 of unit B at δ 4.00. This indicated that units A and B are connected by a

(1→4)-α-D-linkage. N.O.e. patterns arisen on irradiation of H-1 of units B and C at δ 5.03 and 5.08, respectively, did not allow an unambiguous interpretation.

The O-specific polysaccharide was subjected to Smith degradation to give a polymeric product which, judging from its ^{13}C-n.m.r. spectrum (Table II), consisted of the GlcNAc and GalNAc residues. In order to determine the structure of the Smith-degraded polysaccharide, the computer-assisted, ^{13}C-n.m.r.-based method of Lipkind *et al.*[10] was applied. This method involves the evaluation of the spectra for all possible structures of a polysaccharide with the given monosaccharide composition and a search for the structure having the best fit of calculated and experimental spectra. The analysis was performed with the assumption that, usually in bacterial polysaccharides both amino sugars have the D configuration. It revealed only one structure, namely **1**, which was characterized by a relatively small sum of squared deviations of chemical shifts in the calculated and experimental spectra (S = 0.6 per sugar residue). All other possible structures had S values of not less than 2.5 and were, thus, inconsistent with the experimental spectrum.

→6)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→
A B

1

The absolute configurations of the amino sugars were confirmed by calculation of the specific optical rotation value of the Smith-degraded polysaccharide according to Klyne's rule[11]. Only the calculation based on the assumption that both of them were D led to a value of $[\alpha]_D$ close to the experimental value (Table III).

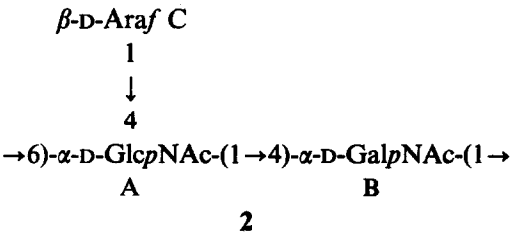
TABLE III

Optical rotation data

Compound	$[\alpha]_D$[a] *(degrees)*	M_r	$[M]_D$ *(degrees)*	*Ref.*
α-D-Glc*p*NAcOMe	+187	235	+439	12
α-D-Gal*p*NAcOMe	+130	235	+305	13
Smith-degraded polysaccharide				
Calculated for				
D-GlcNAc, D-GalNAc	+183	406	+744	
L-GlcNAc, L-GalNAc	−183	406	−744	
D-GlcNAc, L-GalNAc	+33	406	+134	
L-GlcNAc, D-GalNAc	−33	406	−134	
Observed	+233			

[a] For an aqueous solution.

Structure **1** was in accord with the ^{13}C-n.m.r. and n.O.e. data given above and allowed us to conclude that, in the original polysaccharide, the lateral Ara group was attached to O-4 of unit A. Therefore, the O-specific polysaccharide of *P. solanacearum* ICMP 4157 has the structure **2**.



In sugar composition, this polysaccharide differs significantly from O-antigens of *P. solanacearum* strains studied earlier[4,6,14]. It is noteworthy that it contains D-arabinose, which occurs rarely in bacterial lipopolysaccharides. To our knowledge, hitherto D-arabinofuranose has been found[15] only as a component of the O-specific polysaccharide of *Pseudomonas maltophilia* NCIB 9204.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Jasco DIP 360 polarimeter at 25°. Solutions were freeze-dried or evaporated *in vacuo* at 40°. ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 spectrometer for solutions in D_2O at 60° (internal reference, acetone, δ 2.23). ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 spectrometer for solutions in D_2O at 60° (internal reference, acetone, δ 31.45). The standard Bruker software was used to obtain 2D homonuclear (COSY and COSYRCT) and heteronuclear ^{13}C–^{1}H (XHCORRD) shift-correlated spectra (for details, see ref. 16). G.l.c. was performed with a Hewlett–Packard 5890 instrument, equipped with a flame-ionization detector and a glass capillary column (0.2 mm × 25 m), coated with Ultra 1 stationary phase. Gel-permeation chromatography was performed, in a column (70 × 3 cm) of Sephadex G-50, with a pyridine acetate buffer (pH 5.5) and monitoring by the orcinol–H_2SO_4 reaction, or in a column (80 × 1.6 cm) of TSK HW 40 (S) in water with monitoring by a Knauer differential refractometer.

Growth of bacteria and isolation of lipopolysaccharide and O-specific polysaccharide. — The strain of *P. solanacearum* ICMP 4157 was grown, for 36–40 h at 28°, on a synthetic medium N (ref. 17) on a rotatory shaker. Cells were centrifuged off and dried by treatment with acetone and ether.

The lipopolysaccharide was isolated by a standard procedure[7] of phenol–water extraction, followed by removal of nucleic acid by precipitation with Cetavlon and ultracentrifugation at 144 000 *g*. It was cleaved by hydrolysis with 1% acetic acid for 1.5 h at 100°, a lipid residue was removed by centrifugation, and the O-specific polysaccharide was isolated from the supernatant by gel-permeation chromatography on Sephadex G-50.

Acid hydrolysis. — The O-specific polysaccharide (5 mg) was hydrolyzed in a sealed ampoule with 2M trifluoroacetic acid (2 h, 121°). After evaporation, a part of the hydrolyzate was reduced with $NaBH_4$ in water, conventionally acetylated, and analyzed by g.l.c. A second part was treated with KU-2 (H^+) cation-exchange resin in water to

remove amino sugars, the solution was evaporated, and the residue was heated (16 h, 130°) with (*S*)-2-octanol (0.3 mL) in the presence of a drop of trifluoroacetic acid, acetylated, and analyzed by g.l.c.

Smith degradation. — The O-specific polysaccharide (30 mg) was oxidized with 0.1M $NaIO_4$ (1.5 mL) for 24 h at room temperature in the dark, the product was reduced with an excess (30 mg) of $NaBH_4$, desalted by gel-permeation chromatography on TSK HW 40, and hydrolyzed with aq. 1% acetic acid (2 h, 100°) to give the degraded polysaccharide (16 mg), $[\alpha]_D$ + 233° (*c* 2), which was isolated by gel-permeation chromatography on TSK HW 40.

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