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Note

Somatic antigens of pseudomonads: structure of the O-specific polysaccharide of *Pseudomonas fluorescens* biovar A strain IMV 1152

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Pseudomonas fluorescens is a phenotypically heterogeneous microorganism divided into five biovars having a debatable taxonomic rank[1]. In our studies of cell-wall lipopolysaccharides of pseudomonads, aimed at substantiating on a molecular level the biochemical and serological classification of these bacteria (refs. 2–5 and refs. therein), we have established[3] the structure of the O-specific polysaccharide chain of the lipopolysaccharide of *P. fluorescens* biovar G strain IMV 2765, but the relationship of this strain to biovar G is in doubt[6]. Another structurally elucidated O-antigen of this species is that of non-classified strain 361[7,8]. Now we describe the structure of the O-specific polysaccharide of *P. fluorescens* biovar A strain IMV 1152.

The O-specific polysaccharide, $[\alpha]_D + 56°$ (c 0.3, H_2O), was obtained by mild acid degradation of the lipopolysaccharide, which was isolated from bacterial cells by the phenol–water procedure[9].

The ^1H NMR spectrum of the polysaccharide (Fig. 1a and Table I) contained the signals for three anomeric protons at 4.60 (d, $J_{1,2}$ 8.5 Hz), 4.90 (d, $J_{1,2}$ 3.7 Hz), and 4.96 ppm (d, $J_{1,2}$ 3.6 Hz), three CH_3–C groups (H-6 of 6-deoxy sugars) at 0.98, 1.24, and 1.27 ppm (all d, $J_{5,6}$ 5.9–6.2 Hz), three NAc groups at 1.91, 2.00, and 2.02 ppm (all s), and other protons in the region 3.19–4.40 ppm.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 2 and Table II) contained signals for three anomeric carbons at 98.6, 100.2, and 102.5 ppm, three carbons

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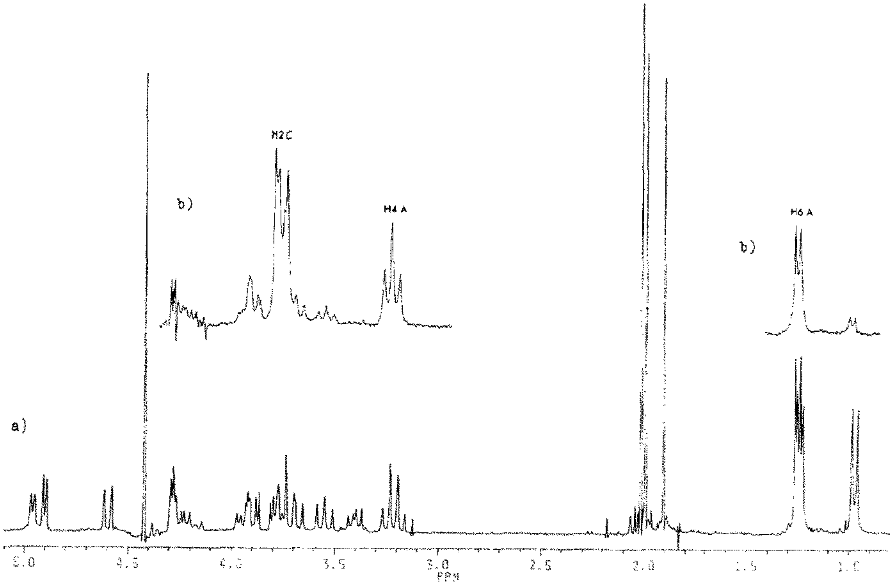

Fig. 1. 250-MHz ^{1}H NMR spectrum of the O-specific polysaccharide (a) and parts of an NOE spectrum with pre-irradiation of H-1 of unit **C** (b).

bearing nitrogen at 54.0, 55.3, and 57.2 ppm, three methyl groups (C-6 of 6-deoxy sugars) at 16.6, 18.0, and 18.2 ppm, other sugar carbons in the region 67.5–84.6 ppm, and three NAc groups (Me at 23.3–23.5 ppm and CO at 175.7 ppm).

Therefore, the polysaccharide has a trisaccharide repeating unit, which contains, most probably, three residues of amino-6-deoxy sugars. Judging[10] from the chemical shifts for CH_3–C groups, one of them has an axial substituent at C-4 (δ 16.6 ppm) and two others an equatorial one (δ 18.0 and 18.2 ppm).

Acid hydrolysis of the polysaccharide revealed the presence of only 2-amino-2,6-dideoxyglucose, which was identified by using an amino acid analyzer. The second amino-6-deoxy sugar was not detected. Solvolysis of the polysaccharide with anhydrous hydrogen fluoride[11] led to two *N*-acetylated amino sugars in the ratio ~ 2 : 1, which were separated by HPLC on an amino phase. Based on ^{1}H (Table I) and ^{13}C NMR data (Table II), they were identified as 2-acetamido-2,6-dideoxyglucose (QuiNAc) and 4-acetamido-4,6-dideoxygalactose (Fuc4NAc), respectively. The ^{13}C NMR spectrum of the former sugar was practically identical to the reported one[12]. Location of the NAc group at C-4 of the latter sugar followed from a low-field position of the signals for H-4α and H-4β at 4.26 and 4.23 ppm, due to deshielding caused by this group, and was confirmed by a high-field position of the signals for C-4α and C-4β at 55.3 and 54.9 ppm, respectively, i.e., in the region expected for carbons bearing nitrogen (see also below). The absence of Fuc4N from the hydrolysate of the polysaccharide is accounted for by the known[13,14] lability of this sugar under acidic conditions.

TABLE I

^{1}H NMR data (δ in ppm, J in Hz)

H-1	H-2	H-3	H-4	H-5	H-6	NAc
2-Acetamido-2,6-dideoxyglucose ($\alpha:\beta$ ~ 1:1)						
*α-Qui*p*NAc*						
5.15	3.90	3.71	3.23	3.93	1.28	2.05
$J_{1,2}$ 3.6	$J_{2,3}$ 10.8	$J_{3,4}$ 9.1	$J_{4,5}$ 9.9	$J_{5,6}$ 6.2		
*β-Qui*p*NAc*						
4.69	3.69	3.48	3.22	3.50	1.31	2.05
$J_{1,2}$ 8.4	$J_{2,3}$ 10.5	$J_{3,4}$ 9.0	$J_{4,5}$ 9.5	$J_{5,6}$ 6.3		
4-Acetamido-4,6-dideoxygalactose ($\alpha:\beta$ ~ 1:2.5)						
*α-Fuc*p4*NAc*						
5.23	3.90	4.02	4.26	4.36	1.10	2.10
$J_{1,2}$ 4.1	$J_{2,3}$ 10.6	$J_{3,4}$ 4.5	$J_{4,5}$ 1.8	$J_{5,6}$ 6.5		
*β-Fuc*p4*NAc*						
4.59	3.34	3.82	4.23	3.93	1.14	2.11
$J_{1,2}$ 8.0	$J_{2,3}$ 10.3	$J_{3,4}$ 4.6	$J_{4,5}$ 1.7	$J_{5,6}$ 6.4		
O-Specific polysaccharide						
→ 4)-α-L-*Qui*p*NAc*-(1 → (*unit* **A**)						
4.90	3.89	3.69	3.23	4.24	1.24	1.91 [a]
$J_{1,2}$ 3.7	$J_{2,3}$ 10.5	$J_{3,4}$ 9.3	$J_{4,5}$ 9.7	$J_{5,6}$ 6.2		
→ 3)-β-D-*Qui*p*NAc*-(1 → (*unit* **B**)						
4.60	3.77	3.54	3.19	3.40	1.27	2.00 [a]
$J_{1,2}$ 8.5	$J_{2,3}$ ~ 10	$J_{3,4}$ ~ 9	$J_{4,5}$ 9.6	$J_{5,6}$ 5.9		
→ 3)-α-D-*Fuc*p4*NAc*-(1 → (*unit* **C**)						
4.96	3.76	3.94	4.28	4.40	0.98	2.02 [a]
$J_{1,2}$ 3.6	$J_{2,3}$ 10.6	$J_{3,4}$ 4.6	$J_{4,5}$ < 2	$J_{5,6}$ 6.2		

[a] Assignments could be interchanged.

The value $[\alpha]_D$ +42° (H_2O) was determined for isolated Fuc4NAc and allowed us to suggest its D configuration. GLC of (+)-2-octyl glycosides of the isolated QuiNAc, as compared with (+)- and (−)-2-octyl glycosides of the authentic sample of D-QuiNAc, indicated[15] the presence of both D and L isomers in the ratio

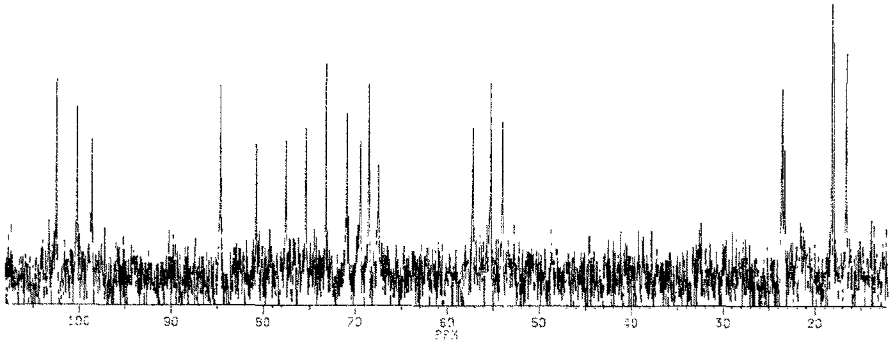

Fig. 2. 75-MHz ^{13}C NMR spectrum of the O-specific polysaccharide (except for the signals for the CO groups).

TABLE II

^{13}C NMR chemical shifts (δ in ppm)[a]

Unit	C-1	C-2	C-3	C-4	C-5	C-6
2-Acetamido-2,6-dideoxyglucose						
α-Qui*p*NAc	91.9	55.6	71.7	76.9	68.8	18.0
β-Qui*p*NAc	96.0	58.2	74.9	76.4	73.2	18.0
4-Acetamido-4,6-dideoxygalactose						
α-Fuc*p*4NAc	93.5	69.8[b]	69.6[b]	55.3	66.4	16.7
β-Fuc*p*4NAc	97.6	73.3[c]	73.1[c]	54.9	71.1	16.7
O-Specific polysaccharide						
→ 4)-α-L-Qui*p*NAc-(1 → (**A**)	98.6	55.3	70.9	84.6	68.5	18.0[d]
→ 3)-β-D-Qui*p*NAc-(1 → (**B**)	102.5	57.2	80.7	75.4	73.1	18.2[d]
→ 3)-α-D-Fuc*p*4NAc-(1 → (**C**)	100.2	69.4	77.5	54.0	67.5	16.6

[a] Additional signals: NAc at 23.1–23.5 ppm (Me) and 175.7–176.7 ppm (CO). [b,c,d] Assignments could be interchanged.

1:1. A small optical activity of the isolated QuiNAc, $[\alpha]_D$ +7.5° (H_2O), was probably accounted for by an impurity.

The ^{1}H NMR spectrum of the polysaccharide was completely assigned with the use of selective spin-decoupling procedures, 2D homonuclear shift-correlated (COSY) spectroscopy, and COSY with one-step relayed coherence transfer (COSYRCT) (Table I). As judged by the values of $^3J_{H,H}$, all sugar residues are pyranoid; two of them have the *gluco* configuration, one being α-linked (unit **A**) and the other β-linked (unit **B**), while the third residue has the *α-galacto* configuration (unit **C**).

Assignment of the ^{13}C NMR spectrum was performed with the aid of sequential, selective heteronuclear ^{13}C–^{1}H double resonance (Table II). The position of the signals for C-2 of units **A** and **B** and C-4 of unit **C** in the region of resonances for carbons bearing nitrogen (54.0–57.2 ppm) proved unequivocally that units **A** and **B** are 2-amino sugars, i.e., QuiNAc, and that unit **C** is a 4-amino sugar, i.e., Fuc4NAc.

The relatively low-field positions of the signals for C-3 of units **B** and **C** and C-4 of unit **A** at 80.7, 77.5, and 84.6 ppm, respectively, as compared to their positions in the spectra of the corresponding free monosaccharides, were due to the α-effects of glycosylation and clarified the substitution pattern of the sugar residues.

Sequence and linkage analysis was performed with the help of 1D NOE spectroscopy with sequential pre-irradiation of H-1 of each of the sugar residues. The following inter-residue contacts, significant for the analysis, were observed: H-1 **A**/H-3 **B**; H-1 **B**/H-3 **C**; H-1 **C**/H-4 **A**. These data confirmed independently the sites of glycosylation, determined from the ^{13}C NMR spectrum, and proved the sequence **A**–**B**–**C** in the linear polysaccharide.

The chemical shift (102.5 ppm) for C-1 of β-Qui*p*NAc (unit **B**) showed that this residue has the same absolute configuration as the linked D-Fuc*p*4NAc (unit ***C***), i.e., the D configuration (a chemical shift of ~ 99 ppm would be expected if these two sugar residues had different absolute configurations[16]). Therefore, the second QuiNAc residue (unit **A**) has the L configuration, which was confirmed independently by the appearance of an NOE response on H-6 of unit **A** as a result of pre-irradiation of H-1 of unit **C** (Fig. 1b). In fact, the spatial proximity of these protons in the preferred conformation is indicative[17] of the different absolute configurations of the constituents in such α-(1 → 4)-linked disaccharides with the *gluco* configuration of the glycosylated sugar residue.

The data obtained showed that the O-specific polysaccharide of *P. fluorescens* biovar A strain IMV 1152 has the following structure:

$$\underset{\textbf{A}}{\rightarrow 4)\text{-}\alpha\text{-L-Qui}p\text{NAc-}(1} \rightarrow \underset{\textbf{B}}{3)\text{-}\beta\text{-D-Qui}p\text{NAc-}(1} \rightarrow \underset{\textbf{C}}{3)\text{-}\alpha\text{-D-Fuc}p\text{4NAc-}(1} \rightarrow$$

This O-antigen contains a rare sugar, 4-acetamido-4,6-dideoxy-D-galactose, which, to the best of our knowledge, has been found before in only two bacterial polysaccharides, namely, in the O-antigen of *Escherichia coli* O10[18,19] and the enterobacterial common antigen[14,20,21], but has not hitherto been isolated and characterised. The simultaneous occurrence of 2-acetamido-2,6-dideoxy-D-glucose and its much less common L isomer has been described[22] for the capsular polysaccharide of *Bacteroides fragilis*, but the structure of the polymer remains obscure.

EXPERIMENTAL

Optical rotations were measured with a Jasco DIP 360 polarimeter for solutions in water at 25°C. Gel chromatography was performed on a TSK HW 40 column (80 × 1.7 cm), using water, with monitoring of the eluate by a Knauer differential refractometer. HPLC was carried out on a column (10 × 250 mm) of Silasorb NH_2 (10 μm) eluted with aq 85% acetonitrile and monitored by a Knauer variable wavelength monitor.

^{1}H NMR spectra were recorded with a Bruker WM-250 instrument for solutions in D_2O at 65°C for the polysaccharide and 25°C for the monosaccharides. ^{13}C NMR spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O at 80°C for the polysaccharide and 25°C for the monosaccharides. Acetone was used as an internal standard (δ_H 2.23 ppm, δ_C 31.45 ppm). Selective spin decoupling, 1D NOE, and 2D homonuclear ^{1}H–^{1}H COSY spectra were obtained as described[23,24].

Growth of bacteria[25], isolation of lipopolysaccharide[4], and isolation of O-specific polysaccharide[26] were performed as described.

Sugar analysis.—The polysaccharide (1 mg) was hydrolysed with 2 M trifluoroacetic acid (100°C, 2 h), and the hydrolysate was analysed by using an amino acid analyzer Biotronik LC-4020, using a column (6 × 220 mm) of Aminex A-5 resin eluted with 0.35 M sodium citrate buffer (pH 5.28) at 65°C.

Another portion of the polysaccharide (20 mg) was treated with anhyd HF for 50 min at ambient temperature, HF was removed in vacuo over solid NaOH, and the residue was subjected to chromatography on TSK HW 40 followed by HPLC separation to give 2-acetamido-2,6-dideoxyglucose, $[\alpha]_D$ +7.5° (c 0.3) {cf.[13] $[\alpha]_D$ +15.8° (H_2O) for 2-acetamido-2,6-dideoxy-D-glucose}, and 4-acetamido-4,6-dideoxy-D-galactose, $[\alpha]_D$ +42° (c 0.5).

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