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Structure of an acidic polysaccharide from the marine bacterium *Pseudoalteromonas flavipulchra* NCIMB 2033^T

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Abstract

An acidic polysaccharide was isolated from *Pseudoalteromonas flavipulchra* type strain NCIMB 2033^T and found to consist of 6-deoxy-L-talose (L-6dTal), D-galactose and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). The identities of the monosaccharides were ascertained by sugar analysis and 1D ^{1}H and ^{13}C NMR spectroscopy in conjunction with 2D COSY, TOCSY, ROESY and ^{1}H, ^{13}C HMQC experiments, which enabled determination of the following structure of the trisaccharide repeating unit of the polysaccharide:

→3)-α-L-6dTal*p*4Ac-(1→3)-β-D-Gal*p*-(1→7)-α-Kdo*p*-(2→

Keywords: Bacterial polysaccharide; Structures; *Pseudoalteromonas flavipulchra*; 6-Deoxy-L-talose; 3-Deoxy-D-*manno*-oct-2-ulosonic acid

Gram-negative bacteria of the genera *Alteromonas* and *Pseudoalteromonas* are aerobic heterotrophic prokaryotes that are widely distributed in the marine environment.[1] These readily cultivatable bacteria have great importance due to their production of a wide range of biologically active compounds such as antibiotics, enzymes, antitoxins, antitumour and antiviral agents.[2] The genus *Pseudoalteromonas* was derived by revision of the genus *Alteromonas*[3] using analysis of 16S rRNA gene sequences.[4] Following enlargement with a number of novel species and reclassification of some of them, this genus currently includes 26 species.

In 1979, six orange-pigmented bacteria were isolated from seawater near Nice, France, and from the surface of seaweed *Ulva lacuta*[5] and assigned to *Pseudoalteromonas aurantia* (former *Alteromonas aurantia*). However, it was noted[5] that one strain, NCIMB 2033 (= ATCC 33042), had some phenotypic features in common with *Pseudoalteromonas piscicida* (former *Pseudomonas piscicida*).[6] Later, based on pheno-chemotaxonomic and genotypic characteristics, it was reclassified[7] into a new separate species *Pseudoalteromonas flavipulchra*.

Structures of cell-surface polysaccharides of a number of *Alteromonas* and *Pseudoalteromonas* strains have been elucidated.[8–10] Distinctive features of the capsular and O-specific polysaccharides of *Pseudoalteromonas* are their acidic character and the presence of unusual sugars and non-sugar substituents. In this paper we report on the structure of a new acidic polysaccharide, isolated from *P. flavipulchra* NCIMB 2033^T, which contains 6-deoxy-L-talose (L-6dTal) and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo).

Bacterial cells were extracted with aqueous 45% phenol,[11] and an acidic polysaccharide was recovered from the aqueous phase by dialysis and studied without further purification.

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Sugar analysis after full acid hydrolysis of the polysaccharide revealed Gal and 6-deoxytalose (6dTal). GLC analysis of the acetylated glycosides with chiral 2-octanol showed that Gal has the D configuration and 6dTal the L configuration. Further studies revealed the presence of an additional monosaccharide, Kdo.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) was typical of a regular polymer. It showed signals for three anomeric carbons at δ 100.3–104.1, one C–CH_2–C group (C-3 of Kdo) at δ 35.4, CH_3–C group (C-6 of 6dTal) at δ 16.4, two HOCH_2–C groups (C-6 of Gal and C-8 of Kdo) at δ 62.1 and 60.8 (data from a DEPT experiment), 12 sugar ring carbons in the region δ 66.8–81.8, one *C*OOH group (C-1 of Kdo) at δ 175.4 and one *O*-acetyl group at δ 21.5 (Me) and 176.3 (*C*O). The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons at a lower field than δ 82.0 demonstrated the pyranose form of all sugar residues.[12]

Accordingly, the ^1H NMR spectrum of the polysaccharide contained in the low-field region signals for two anomeric protons (H-1 of Gal and 6dTal) at δ 5.12 and 4.63 and H-4 of a 4-*O*-acetylated 6dTal residue at δ 5.38 (see below). In the high-field region, there were signals for the C–CH_2–C group of Kdo (H-3ax at δ 1.76 and H-3eq at δ 2.18), CH_3–C group of 6dTal (H-6, 3 H) at δ 1.12 and an *O*-acetyl group at δ 2.22.

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, ROESY and H-detected ^1H, ^{13}C HMQC experiments (Tables 1 and

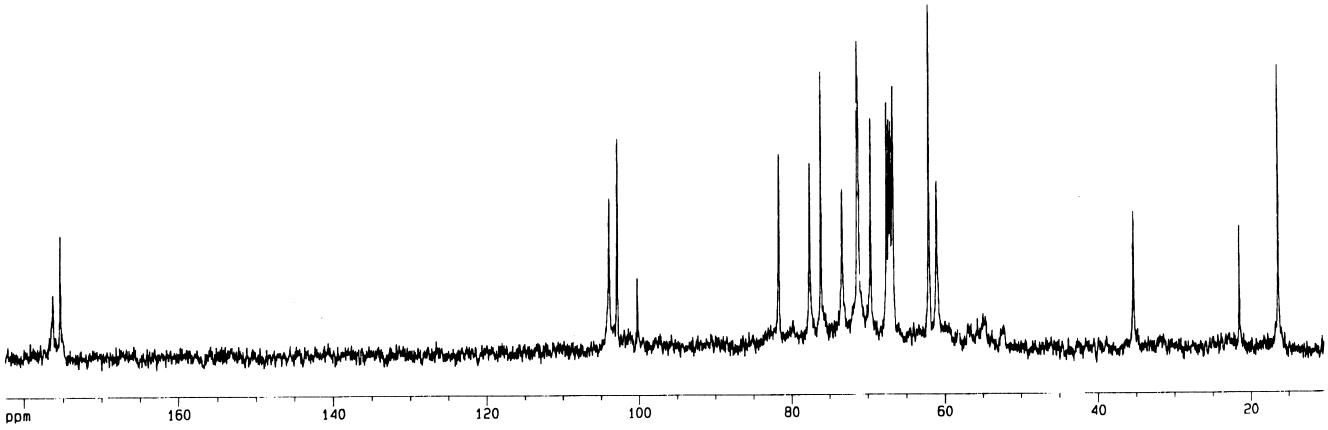

Fig. 1. 125-MHz ^{13}C NMR spectrum of the polysaccharide from *P. flavipulchra*.

Table 1
^{13}C NMR data of the polysaccharide (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
→3)-α-L-6dTal*p*4Ac-(1→	104.1	67.6	67.1	73.4	67.3	16.4		
→3)-β-D-Gal*p*-(1→	102.9	71.4	81.8	69.7	76.2	62.1		
→7)-α-Kdo*p*-(2→	175.4	100.3	35.4	66.8	66.8	71.2	77.7	60.8

The chemical shifts for *O*Ac are δ 21.5 (Me) and 176.3 (CO).

Table 2
^1H NMR data of the polysaccharide (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	
→3)-α-L-6dTal*p*4Ac-(1→	5.12	3.97	4.31	5.38	4.24	1.12	
→3)-β-D-Gal*p*-(1→	4.63	3.74	3.74	3.96	3.74	3.76	
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8
→7)-α-Kdo*p*-(2→	1.76	2.18	3.95	4.18	4.06	4.10	3.98

The chemical shift for *O*Ac is δ 2.22.

2). The spin systems for β-Gal and α-6dTal were identified by correlations of H-1 with H-2,3,4 in the TOCSY spectrum. The ROESY spectrum showed H-1,H-5 and H-4,H-5 correlations for β-Gal and H-1,H-2, H-4,H-5 and H-4,H-6 correlations for α-6dTal. For the Kdo residue, the COSY and TOCSY spectra correlated H-3ax with H-4,5 and H-6 with H-7,8.

The β configuration of Gal and the α configuration of 6dTal were confirmed by the C-5 chemical shifts of 76.2 and δ 67.3 (compare published data δ 71.7 and 76.3 for α- and β-Gal*p*;[13] δ 67.7 and 72.0 for α- and β-6dTal*p*,[14] respectively). The anomeric configuration of Kdo was inferred based on the ^{1}H NMR chemical shifts δ 1.76 and 2.18 for H-3ax and H-3eq, which are typical of the α configuration (δ 1.70 and 2.46 would be expected for H-3ax and H-3eq of β-Kdo*p*, respectively[15]), and confirmed by the comparison of the C-4,5,6 chemical shifts with published data for methyl 3-deoxy-α- and -β-*manno*-oct-2-ulopyranosonate.[16]

Relatively low-field positions of the signals for C-3 of Gal and 6dTal and C-7 of Kdo at δ 81.8, 67.1 and 77.7, respectively, as compared with their positions in the corresponding non-substituted monosaccharides at δ 74.1,[13] 66.3[14] and 70.5,[16] showed the modes of glycosylation of the monosaccharides. A smaller than expected down-field displacement for C-3 of 6dTal could be accounted for by *O*-acetylation of this sugar at position 4. This was demonstrated by a low-field position at δ 5.38 of the H-4 signal, which was caused by a deshielding effect of the *O*-acetyl group (compare with the chemical shifts δ 3.93 and 3.76 for H-3 and H-4 in the non-acetylated α-6dTal*p*[14] and *O*-deacetylated polysaccharide studied, respectively). The degree of *O*-acetylation was estimated to be close to 100%.

The 2D ROESY spectrum showed interresidue 6dTal H-1,H-3 Gal and Gal H-1,Kdo H-7 cross-peaks at δ 5.12/3.74 and 4.63/4.10, respectively. A Kdo H-3ax/6dTal H-2 cross-peak at δ 1.76/3.97 confirmed the linkage between the two sugars. These data are consistent with the glycosylation pattern and defined the sequence of the monosaccharide residues in the repeating unit. Therefore, the polysaccharide of *P. flavipulchra* NCIMB 2033^T has the following structure:

→3)-α-L-6dTal*p*4Ac-(1→3)-β-D-Gal*p*-(1→7)-

α-Kdo*p*-(2→

Although less common than L-rhamnose and L-fucose, L-6dTal occurs in a number of bacterial polysaccharides and is often present in an *O*-acetylated form.[17] Kdo is also a rare component of bacterial polysaccharides; previously, it has been found in an acidic polysaccharide of *Pseudoalteromonas* (*Alteromonas*) *nigrifaciens* IAM 13010^T.[15]

1. Experimental

1.1. Bacterial strain, growth and isolation of the polysaccharide

P. flavipulchra NCIMB 2033^T was grown on the modified Youschimizu–Kimura medium.[18] Wet bacterial cells were extracted with hot aq 45% phenol,[11] the resulting mixture was centrifuged, the aqueous layer dialysed, freed from insoluble contaminations by centrifugation, concentrated in vacuum and freeze-dried to yield a polysaccharide (650 mg from 20 L cultural fluid).

1.2. *O*-Deacetylation of the polysaccharide

The polysaccharide (15 mg) was treated with aq 12.5% NH_3 at 37 °C for 16 h, and the *O*-deacetylated polysaccharide (10 mg) was isolated by GPC on a column (90 × 2.5 cm) of TSK HW-40 (S) (Supelco) in aq 1% HOAc monitored with a Knauer differential refractometer.

1.3. Chemical analyses

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Neutral sugars were identified by GLC of the alditol acetates on a Hewlett-Packard 5890 instrument equipped with a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 150–290 °C at 3 °C/min. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (−)-2-octyl glycosides according to the published method.[19,20]

1.4. NMR spectroscopy

Samples were neutralised to pH 7.0 with aq 12.5% NH_3 and deuterium-exchanged by freeze-drying from D_2O. NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer equipped with an SGI INDY workstation for solutions in 99.96% D_2O at 24 °C, using internal C_3H_6O (δ_H 2.225, δ_C 31.45) as reference. The data were acquired and performed using XWINNMR 2.1 software. A mixing time of 200 and 100 ms was used in TOCSY and ROESY experiments, respectively; other 2D NMR parameters were essentially the same as described previously.[9,10]

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