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Carbohydrate Research 313 (1998) 61–64

CARBOHYDRATE RESEARCH

Note

Structure of the O-specific polysaccharide from *Pseudoalteromonas elyakovii* sp. nov. CMM 162

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Received 21 May 1998; accepted 28 August 1998

Abstract

The structure of the O-specific polysaccharide from lipopolysaccharide of *Pseudoalteromonas elyakovii* sp.nov. CMM 162 on the basis of NMR data, Smith degradation and methylation study was elucidated as follows: →2)-α-D-Glc*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-NAc-(1→6)-α-D-Glc*p*-(1→

Keywords: Pseudoalteromonas elyakovii sp.nov.; NMR spectroscopy; Methylation; Smith degradation

In accordance with the recent phylogenetic analysis of the genera *Alteromonas*, *Shewanella* and *Moritella* [1], the genus *Alteromonas* was split into two genera. A newly created genus *Pseudoalteromonas* accommodated 11 species that were previously *Alteromonas* species whereas genus *Alteromonas* was restricted to a single species *Alteromonas macleodii*. The strain CMM 162 was isolated from the coelomic fluid of the far-eastern mussel *Crenomytilus grayanus*. The strain was assigned to genus *Pseudoalteromonas* on the basis of its phenotypic and genotypic characteristics. The G+C ratio of the DNA is 38.9%. The CMM 162 strain significantly differs from other phenotypically similar species, *P. haloplanktis*, *P. tetraodonis*, *P. atlantica*, and *P. carrageenovora* by utilization of carbon sources, low DNA relatedness (20–40%) and its antigenic specificity as well. The name *Pseudoalteromonas elyakovii* is proposed for the new species [2].

The structures of the antigenic polysaccharides from five strains of *Pseudoalteromonas (Alteromonas)* spp. have been established earlier [3–7]. In the present paper, we report the results of structural analysis of the O-specific polysaccharide from *P. elyakovii* sp. nov. CMM 162 from the Collection of Marine Microorganisms (CMM) which is at the disposal of our Institute.

1. Results and discussion

Electron microscopy has shown that *P. elyakovii* is capsulated [2]. The capsular polysaccharide (CPS) was obtained from bacterial cells by extraction with 2% aqueous saline using ultrasonication and purification by anion-exchange

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0008-6215/98/$—see front matter
PII S0008-6215(98)00235-3

chromatography. Sugar analysis of the CPS by PC and GLC of derived alditol acetates revealed the presence of Rha and Gal residues. The ^{13}C NMR spectrum of CPS also indicated the presence of an *O*-acetyl group (signal at 21.4 ppm) and pyruvate (signal Me group at 26.3 ppm).

Lipopolysaccharide (LPS) was obtained from bacterial cells by hot phenol–water extraction [8]. Treatment of LPS with dilute acetic acid (1%, 100 °C, 2 h) followed by size-exclusion chromatography of the water-soluble fraction on the TSK HW 50(F) gel gave the O-specific polysaccharide (PS). Sugar analysis of PS by PC and GLC of the corresponding alditol acetates showed the presence of D-Glc, D-Gla, and D-GalNAc residues in ratios 2:1:2. All sugars were isolated by preparative PC, high-voltage electrophoresis and its $[\alpha]_D$ values indicated the D-configuration of these monosaccharides.

The ^{13}C NMR spectrum (Table 1) indicated a pentasaccharide repeating unit (signals of five anomeric carbons at 96.7, 96.8, 99.5, 103.3 and 104.4 ppm). There were also present two N-acetyl signals (CH_3) at 23.5 and 23.7 ppm, (CO) at 175.0 and 176.1 ppm, two carbons bearing nitrogen at 52.0 and 53.5 ppm, one signal of the substituted CH_2OH-group at 67.9 ppm and 22 signals of the secondary carbon atoms. Coupling constant values (173–174 Hz) for the signals at 96.7, 96.8 and 99.5 ppm and 160 Hz for the signals at 103.3 and 104.4 ppm demonstrated [9] the pyranose form of each sugar; three of them possessed α-configuration and two were β-glycosidic residues. Methylation of PS by the Hakomori method [10] followed by hydrolysis, borohydride reduction, acetylation and analysis of the methylated sugars by GLC–MS demonstrated the presence of 2- and 6-substituted Glc residues, 3-substituted Gal residue and 3- and 4-substituted GalNAc residues. Smith degradation of PS followed by borohydride reduction, mild acid hydrolysis and size exclusion chromatography furnished oligosaccharide (OS). Sugar analysis of OS by PC and GLC of alditol acetates indicated the presence of D-Gal, D-GalNAc and glycerol in the ratios 1:2:1. Further structural analysis of OS was carried out using ^1H and ^{13}C NMR spectroscopy (Table 2). A complete assignment of the signals in the ^1H NMR spectrum was achieved using homonuclear resonance in a difference mode [11], 2D homonuclear shift-correlated spectroscopy (COSY) and two-step relayed coherence transfer spectroscopy (COSY RCT). As a result, the chemical shifts [12] and $J_{H,H}$ values [13] confirmed the pyranose form of sugar residues. One sugar residue was determined to possess the α-*galacto*-configuration ($J_{1,2}$ 3.5 Hz, $J_{2,3}$ 10 Hz, $J_{3,4} < 2$ Hz) and two β-*galacto*-configuration ($J_{1,2}$ 8.0 Hz, $J_{2,3}$ 10 Hz, $J_{3,4} < 2$ Hz). The ^{13}C NMR spectrum of OS was completely assigned using 2D heteronuclear $^{13}C/^1H$ shift-correlated spectroscopy (Table 2) and the structure of the OS was established as follows:

$$\beta\text{-D-Gal}p\text{NAc-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}$$
$$(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1 \rightarrow 1)\text{-Gro}$$

The ^{13}C NMR spectrum of the PS was independently assigned by computer-assisted ^{13}C NMR analysis by the method of [14]. In this case, practically full coincidence of the chemical shift values in the experimental and calculated spectra was observed (Table 1). The sum of squared deviation of the chemical shifts was 0.1. On the basis of methylation data as well as NMR spectroscopy

Table 1
^{13}C NMR data for PS *P. elyakovii* CMM 162 (δ, ppm)[a]

Sugar residue	Carbon atoms					
	C-1	C-2	C-3	C-4	C-5	C-6
→2)-α-D-Glc*p*-(1→	99.5	76.7	72.7	70.9	73.2	61.9
	(99.5)[b]	(76.7)	(72.7)	(70.9)	(73.2)	(61.9)
→4)-β-D-Gal*p*NAc-(1→	104.4	53.5	72.2	77.7	76.6	61.5
	(104.4)	(53.3)	(72.2)	(77.7)	(76.6)	(61.5)
→3)-α-D-Gal*p*-(1→	96.8	68.9	80.1	70.9	72.2	62.4
	(96.8)	(68.9)	(80.4)	(70.3)	(72.2)	(62.4)
→3-β-D-Gal*p*NAc-(1→	103.3	52.0	77.3	65.4	76.4	62.2
	(103.3)	(52.0)	(77.3)	(65.4)	(76.4)	(62.2)
→6)-α-D-Glc*p*-(1→	96.7	72.7	74.0	70.9	72.2	67.9
	(96.7)	(72.7)	(74.0)	(70.9)	(72.2)	(67.9)

[a]Additional signals assigned to NHAc: 23.5, 23.7 ppm (2 Me) and 175.0; 176,1 ppm (2 CO).
[b]Calculated data are given in parentheses.

Table 2
NMR data for OS from PS *P. elyakovii* CMM 162 (δ, ppm)[a,b,c]

	$^1H/^{13}C$					
Sugar residue	1	2	3	4	5	6
β-D-Gal*p*NAc-(1→	4.63	3.94	3.74	3.95	3.68	3.7–3.9
	104.4	53.75	72.1	69.0	76.1[d]	61.9
→3)-α-D-Gal*p*-(1→	5.11	3.91	3.80	4.20	3.86	3.7–3.9
	96.5	68.2	80.3	70.2	72.1	62.2
→3)-β-D-Gal*p*NAc-(1→	4.58	4.09	3.82	4.17	3.68	3.7–3.9
	102.8	51.85	76.5	65.1	75.9[d]	62.2
→1)-Gro	4.05	3.72	3.65–3.70			
	3.77					
	72.0	73.0	63.8			

[a]Internal reference acetone (δ_H 2.225 ppm, δ_C 31,45 ppm).
[b]Coupling constants for sugar residues: $J_{1,2}$ 3.5 Hz (α-anomer) and 8.0 Hz (β-anomer), $J_{2,3}$ 10 Hz, $J_{3,4}$ 3.5 Hz, $J_{4,5} < 2$ Hz.
[c]Additional signals assigned to NHAc: 23.5 ppm (2 Me) and 175.0 ppm (2 CO).
[d]Tentative assignment.

data including computer-assisted ^{13}C NMR analysis by the method [14], the structure of the O-specific polysaccharide from *P. elyakovii* CMM 162 was established:

→ 2)-α-D-Glc*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-
α-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→6)-
α-D-Glc*p*-(1→

2. Experimental

General methods.—PC, GLC, GLC–MS, electrophoresis, size-exclusion chromatography and methylation of the PS were performed as described earlier [3].

NMR spectroscopy.—NMR spectra were recorded on a Bruker WM-250 and AM-300 spectrometers for solutions in D_2O (internal acetone, δ_H 2.225 ppm, δ_C 31.45 ppm) as described [7]. The standard Bruker software was used to obtained 2D spectra.

Bacterial strain, growth and isolation of the LPS and O-specific polysaccharide (PS).—*P. elyakovii* strain CMM 162 was grown in the Youschimizu–Kimura medium (30 L) [15]. For the isolation of the capsular polysaccharide the wet bacterial cells were extracted twice with 2% NaCl using ultrasonication (44 kHz, 5 min, 4 °C), cells were removed by centrifugation and extracted with hot aq 45% phenol for the obtaining of the LPS [8]. Nucleic acids were precipitated with 50% tricloroacetic acid at pH 2, LPS was purified by ultra centrifugation at 105,000×*g* (650 mg). LPS (500 mg) was hydrolyzed with aq 1% CH_3COOH (50 ml, 100 °C, 2 h), lipid A precipitate (100 mg) was removed by centrifugation and the supernatant was concentrated in vacuo and precipitated with ethanol (5 vols). The 3-deoxy-D-*manno*-octulosonic acid (KDO) was identified in ethanol solution by PC and paper electrophoresis with the authentic sample. The PS fraction was chromatographed on Sephadex G-50 to afford O-specific PS (260 mg) and low-molecular mass substance (40 mg).

Sugar analysis.—Hydrolysis of the PS-s (1 mg) was performed with 2 M CF_3COOH (1 mL, 120 °C, 2 h), after borohydride reduction and full acetylation, alditol acetates derived were analysed by GLC and GLC–MS. In a preparative scale, the PS (20 mg) was hydrolysed with 2 M HCl (10 mL, 120 °C, 3 h). The sugars were isolated by preparative PC and paper electrophoresis and identified as: D-Glc $[\alpha]_D + 90°$ (C 1.0, H_2O), D-Gal $[\alpha]_D + 75°$ (C 0.5, H_2O), D-GalN.HCl $[\alpha]_d + 97°$ (C 0.2, H_2O) (cf. corresponding values for D-Glc, D-Gal and D-GalN.HCl (+52.7°, +80.2°, +80° [16])

Smith degradation of PS.—A solution of PS (70 mg) in 0.1 M $NaIO_4$ (5 mL) was kept in the dark for 70 h at room temprature and the product was reduced conventionally with $NaBH_4$ for 4 h. The excess of borohydride was destroyed with acetic acid, the mixture was dialysed, and the solution was concentrated. The polyalcohol obtained was hydrolysed with aq 1% CH_3COOH at 100 °C for 2 h, the hydrolysate was concentrated, and the residue was reduced with $NaBH_4$ and chromatographed on TSK HW-40 (F) gel to yield of the oligosaccharide (OS) (12 mg) $[\alpha]_D + 60°$ (CO 1.2, H_2O).

Acknowledgements

This work was partially supported by grant no. 96-04-49028 of the Russian Foundation for Basic Research.

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