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Structure of the O-Specific Polysaccharide of the Marine Bacterium *Arenibacter palladensis* KMM 3961^T Containing 2-Acetamido-2-deoxy-L-galacturonic Acid

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Abstract—The O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of the marine bacterium *Arenibacter palladensis* type strain KMM 3961^T and studied by chemical methods and ^{1}H and ^{13}C NMR spectroscopy including 2D COSY, TOCSY, ^{1}H,^{13}C HSQC, and HMBC experiments. The polysaccharide was shown to consist of tetrasaccharide repeating units containing two mannose residues (Man), one 2-acetamido-2-deoxy-D-galactose residue (D-GalNAc), and one 2-acetamido-2-deoxy-L-galacturonic acid residue (L-GalNAcA) and having the following structure:

→2)-α-D-Man*p*-(1→6)-α-D-Man*p*-(1→4)-α-L-Gal*p*NAcA-(1→3)-β-D-Gal*p*NAc-(1→.

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Key words: *Arenibacter palladensis*, marine bacteria, O-specific polysaccharide, structure, NMR spectroscopy, 2-acetamido-2-deoxy-L-galacturonic acid

The genus *Arenibacter* belonging to the family Flavobacteriaceae [1] now involves six species: *A. latericius*, *A. troitsensis*, *A. certesii*, *A. palladensis*, *A. echinorum*, and *A. nanhaiticus* [2-7]. Microorganisms of the genus are Gram-negative, aerobic, heterotrophic and dark-orange-pigmented marine bacteria isolated from various environments, including bottom-sediment samples, the brown alga *Chorda filum*, the green alga *Ulva fenestrata*, the edible holothurian *Apostichopus japonicus*, and the sea urchin *Strongylocentrotus intermedius*. No information on the composition and structure of the polysaccharides of *Arenibacter* marine bacteria had been published until now. Only data on the structural characterization of the carbohydrate backbone of the lipooligosaccharide of the *A. certesii* strain KMM 3941^T has been reported [8].

In this paper we present our results on structural investigation of the O-specific polysaccharide (OPS) of the marine bacterium *A. palladensis* type strain KMM 3961^T.

MATERIALS AND METHODS

The microorganism *A. palladensis* type strain KMM 3961^T isolated from the green alga *U. fenestrata*, which was collected in Pallada Bay, Gulf of Peter the Great, Sea of Japan, was taken from the Collection of Marine Microorganisms of the Pacific Institute of Bioorganic Chemistry. The bacteria were cultured for 48 h at room temperature in culture medium containing in 1 liter of a mixture of sea water and distilled water (1 : 1), 5 g the Bacto peptone (Difco, USA), 2 g the Bacto yeast extract (Difco), 1 g glucose, 0.02 g KH_2PO_4, and 0.05 g $MgSO_4 \cdot 7\ H_2O$, pH 7.6-7.8.

The lipopolysaccharide was isolated from dry bacterial cells by hot phenol–water extraction [9] and purified by precipitation of nucleic acids and protein with cold 50% aqueous trichloroacetic acid at pH 2.0. The precipitate was removed by centrifugation; the supernatant was dialyzed against distilled water and lyophilized. The lipopolysaccharide was isolated in a yield of 3.4% of dry bacterial cell mass.

Abbreviations: COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; MS, mass spectrometry; OPS, O-specific polysaccharide; TOCSY, total correlation spectroscopy.

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The lipopolysaccharide (250 mg) was degraded with aqueous 2% acetic acid at 100°C for 4 h until the precipitation of lipid A, which was removed by centrifugation (12,000*g*, 30 min). The supernatant was successively fractionated on TSK HW-50 (F) (TOYO SODA, Japan) and TSK HW-40 (F) (TOYO SODA) gels in 0.3% acetic acid and H_2O, respectively. The eluent was monitored with a RIDK 101 differential refractometer (Czechia). The OPS was obtained in a yield 46% of the lipopolysaccharide mass.

Monosaccharides were analyzed by GLC and GLC-mass spectrometry (MS) of (i) the alditol acetates after hydrolysis of the polysaccharide (1 mg) with 1 M trifluoroacetic acid at 100°C for 4 h followed by acetylation and of (ii) the acetylated methyl glycosides obtained by methanolysis of the polysaccharide (1 mg) with 1 M HCl in methanol (100°C, 3 h) and subsequent acetylation.

The polysaccharide was methylated with methyl iodide in dimethylsulfoxide in the presence of sodium methylsulfinylmethanide according to a published procedure [10]. The methylated polysaccharide was converted into acetylated partially methylated methyl glycosides by methanolysis with 1 M HCl in methanol (100°C, 3 h) followed by acetylation and analyzed by GLC-MS.

GLC was performed on a Agilent 6850 chromatograph (USA) equipped with a HP 5 MS capillary column (0.25 mm × 30 m) with 5% Phenyl Methyl Siloxane phase in a temperature gradient from 150 to 230°C (10 min) at 3°C/min for sugar analysis and from 110 to 230°C (10 min) at 3°C/min for analysis of partially methylated monosaccharides. GLC-MS was performed on a Hewlett-Packard 5890 chromatograph (USA) equipped with a HP 5 MS capillary column with 5% Phenyl Methyl Siloxane stationary phase and connected with a Hewlett-Packard 5973 mass spectrometer (USA) using the same chromatographic conditions as in GLC.

The polysaccharide (30 mg) was selectively cleaved with cold trifluoromethanesulfonic acid (1 ml) at −5°C for 3 h under anhydrous conditions [11]. After neutralization with aqueous 25% ammonia at 0°C to pH 7, the reaction products were desalted and fractionated by gel-permeation chromatography on TSK HW-40 (F) to give disaccharide (5 mg). The specific optical rotation of the isolated disaccharide was $[\alpha]_D^{20}$ −40° (0.5 g per 100 ml water).

The absolute configuration of the mannose residue was determined by measuring of the specific optical rotation on a Perkin Elmer model 343 instrument. Free monosaccharide (4 mg) was isolated from polysaccharide hydrolyzate (30 mg) using descending preparative paper chromatography performed on Filtrak FN-12 paper in butan-1-ol–pyridine–water (6 : 4 : 3 v/v). The specific optical rotation of the isolated Man $[\alpha]_D^{20}$ +12° (0.4 g per 100 ml water) indicated its D-configuration; the literature gives the specific optical rotation for D-Man as $[\alpha]_D^{20}$ +14° and L-Man as $[\alpha]_D^{20}$ −14° [12].

NMR spectra were recorded on a Bruker DRX-500 spectrometer (Germany) in 99.96% D_2O solutions at 30°C using acetone as an internal standard (δ_C 31.45, δ_H 2.22). The samples were preliminarily lyophilized from D_2O. Two-dimensional spectra were recorded using standard Bruker software (Germany); data were acquired and processed using the XWINNMR 2.1 program. A mixing time of 200 msec was used in TOCSY experiment.

RESULTS AND DISCUSSION

The O-specific polysaccharide (OPS) from *A. palladensis* was obtained by mild acid degradation of the lipopolysaccharide isolated from dried bacterial cells by extraction with aqueous phenol [9] followed by gel-permeation chromatography. Sugar analysis by GLC and GLC-MS analysis of the O-acetylated methyl glycosides and alditol acetates revealed that residues of Man, GalN, and hexosaminuronic acid are involved in the composition of the OPS.

The absolute D-configuration of the Man residue was defined on the basis of the value of its specific optical rotation. The absolute configuration of the other monosaccharides and exact identification of hexosaminuronic acid were established by NMR spectroscopy.

The ^{13}C NMR spectrum of the OPS (Fig. 1) contained signals for four anomeric carbons at δ 98.6, 99.8, 101.2, and 102.6, two unsubstituted hydroxymethyl groups (C6 of GalN and one Man residue) at δ 62.2 and 63.1, two nitrogen-bearing carbons of amino sugars at δ 50.9 and 53.0, one carboxyl group (C6 of HexNA) at δ 174.1, two N-acetyl groups at δ 23.7 and 23.3 (both CH_3) and 175.4 and 175.6 (CO). The DEPT-135 experiment showed that the signal at δ 66.6 belongs to C6 of the substituted second Man residue, and the other atoms of monosaccharide cycles are in the area δ 69.0-77.2. The absence of resonances at δ 82-85 characteristic of the spectrum of furanose cycles showed that all of the monosaccharides are in the pyranose form [13].

As follows from the ^{13}C NMR spectrum of the OPS taken without suppression of protons, the signals for anomeric carbon atoms at δ 102.6, 98.3 and 99.8 displayed $^1J_{C1\text{-}H1}$ coupling constants of 173.6, 170.7 and 172.0 Hz; hence, they belonged to the α-linked sugars. The fourth C1 signal at δ 101.6 was characterized by lower $^1J_{C1\text{-}H1}$ coupling constant value of 160.3 Hz, thus indicating that the remaining monosaccharide is β-linked [14].

The ^1H NMR spectrum of the polysaccharide, besides signals for four anomeric protons in the area of δ 4.51-5.25, contained signals for H4 and H5 of GalNAcA, two N-acetyl groups at δ 2.03, and the other protons of monosaccharides in the area of δ 3.50-4.17.

Methylation analysis of the OPS resulted in identification of 3,4,6-tri-O-methyl mannose, 2,3,4-tri-O-

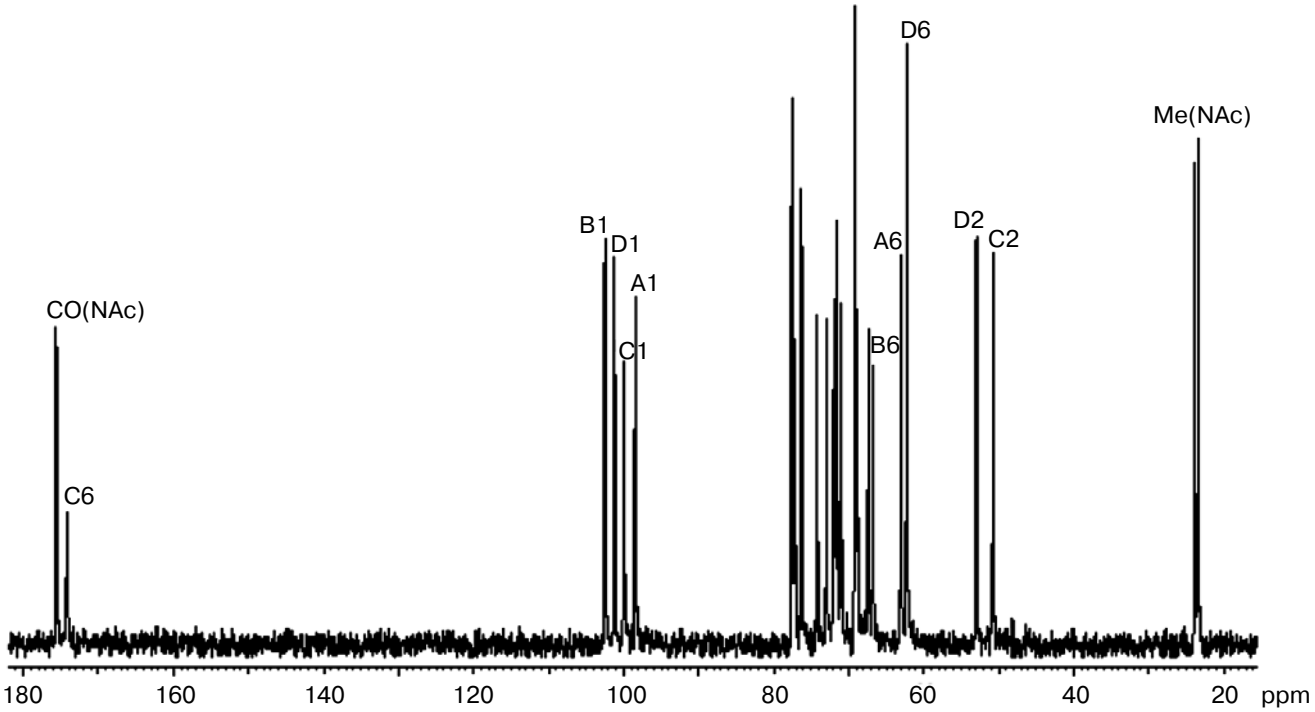

Fig. 1. The ^{13}C NMR spectrum of the O-specific polysaccharide of *A. palladensis* KMM 3961^T. Arabic numerals refer to the carbons in the sugar residues denoted as described in the table.

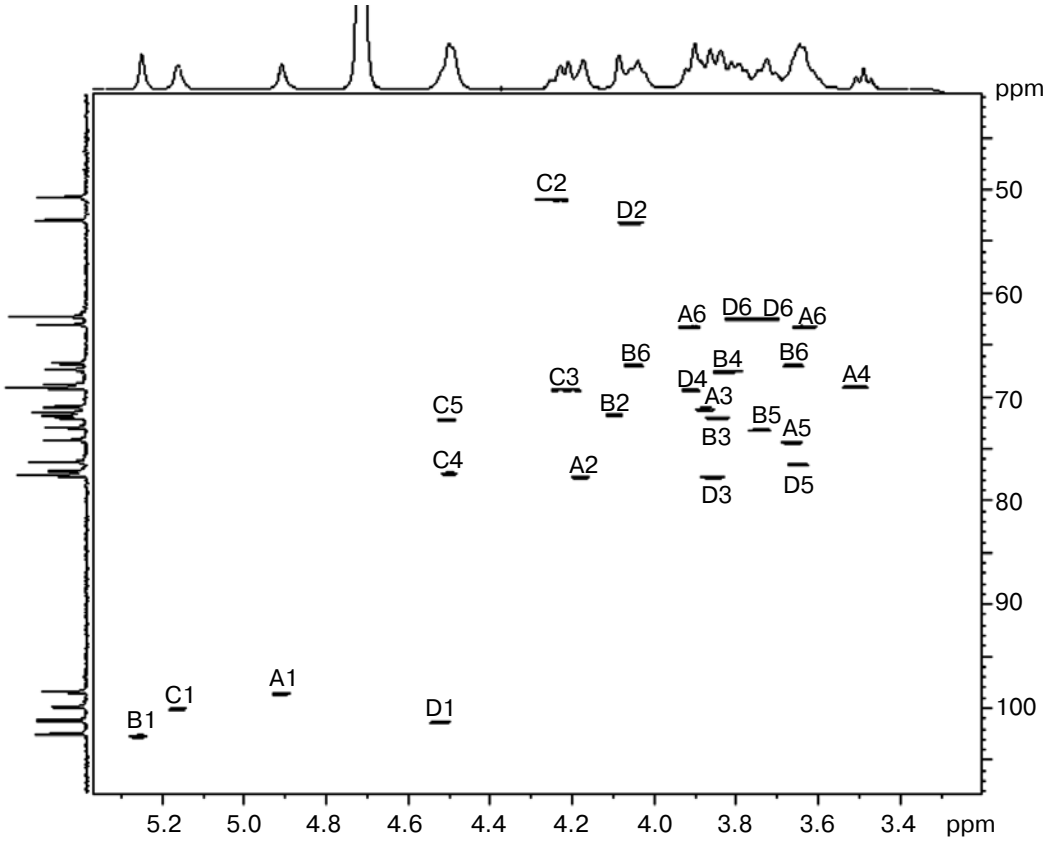

Fig. 2. Part of the 2D ^{1}H,^{13}C HSQC spectrum of the OPS of *A. palladensis* KMM 3961^T. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals refer to the atoms in sugar residues denoted by capital letters as shown in the table.

Data of ^{1}H and ^{13}C NMR spectra of O-specific polysaccharide and disaccharide of *A. palladensis* KMM 3961^{T} (δ, ppm)

Sugar residue	H/C	1	2	3	4	5	6 (6a,6b)
O-Specific polysaccharide*							
→2)-α-D-Man*p*-(1→ A	^{1}H	4.91	4.17	3.88	3.50	3.66	3.62, 3.90
	^{13}C	98.6	77.1	71.6	69.0	74.4	63.1
→6)-α-D-Man*p*-(1→ B	^{1}H	5.25	4.08	3.84	3.80	3.74	3.66, 4.04
	^{13}C	102.6	71.5	72.2	67.6	73.2	66.6
→4)-α-L-Gal*p*NAcA-(1→ C	^{1}H	5.16	4.23	4.21	4.48	4.48	
	^{13}C	99.8	50.9	69.3	77.2	72.1	174.1
→3)-β-D-Gal*p*NAc-(1→ D	^{1}H	4.51	4.03	3.86	3.90	3.63	3.78, 3.71
	^{13}C	101.2	53.0	76.4	71.1	76.3	62.2
Disaccharide**							
α-L-Gal*p*NAcA-(1→	^{1}H	5.12	4.18	4.00	4.34	4.45	
	^{13}C	99.8, 100.0	50.5	68.7	70.7	72.7	175.1
→3)-α-D-Gal*p*NAc	^{1}H	5.15	4.31	3.69	3.98	4.34	3.70, 3.70
	^{13}C	92.4	50.5	74.5	69.7	70.7	62.3
→3)-β-D-Gal*p*NAc	^{1}H	4.62	4.02	3.84	3.90	3.69	3.73, 3.73
	^{13}C	96.7	54.0	77.6	69.0	76.3	62.1

* Chemical shifts for *N*-acetyl groups are: δ_H 2.03; δ_C 23.7-23.2 (Me) and 175.6-175.4 (CO).
** Chemical shifts for *N*-acetyl groups are: δ_H 2.04, 2.03, 2.00; δ_C 23.4, 23.2 (Me) and 175.5 (CO).

methyl mannose, and 2-deoxy-4,6-di-O-methyl-2-(N-methyl)acetamidogalactose. A low amount of 2,3,4,6-tetra-O-methyl mannose indicates that the mannose residue is on a terminal end of the repeating unit.

Thus, the polysaccharide consists of linear repeating tetrasaccharide units containing two D-Man residues, one of them substituted in position 2 and the other in position 6, one residue each of GalNAc substituted in position 3, and HexNAcA (hexosaminuronic acid).

For further structural analysis, ^{1}H and ^{13}C NMR spectra of the OPS were assigned (table) using two-dimensional correlation spectroscopy, including $^{1}H,^{1}H$ COSY, TOCSY, $^{1}H,^{13}C$ HSQC, and HMBC experiments.

The COSY and TOCSY spectra revealed correlation peaks between H1 and H2 and between H2 and other protons for both residues with the *manno*-configuration (Man[1] and Man[2]), whose anomeric protons are at δ 4.91 and 5.25. Monosaccharide residues of the *galacto*-configuration (GalNAc and GalNAcA), whose anomeric protons are at δ 4.51 and 5.16, were identified by the presence of correlation peaks of the H1 proton with the H2-H3 protons of spin systems of both monosaccharides in the TOCSY spectrum of the OPS. Protons H4-H6 for GalNAc and H4-H5 for GalNAcA were identified by using 2D $^{1}H,^{13}C$ HSQC and HMBC spectra.

The ^{13}C NMR spectrum of the OPS was interpreted using the two-dimensional $^{1}H,^{13}C$ HSQC experiment (table). The positions of amino groups in the GalNAc and GalNAcA were confirmed according to correlation of H2 protons resonating at δ 4.03 and 4.23 with the corresponding carbon atoms linked with nitrogen (signals at δ 53.0 and 50.9). A fragment of the two-dimensional $^{1}H,^{13}C$ HSQC spectrum of the OPS is given in Fig. 2.

The signal for C6 of GalNAcA residue was identified by correlation H4/C6 in the $^{1}H,^{13}C$ HMBC spectrum. A study of the ^{13}C NMR spectrum of the OPS before and after of acidification to pH 2 unambiguously determined the signal for C5 of GalNAcA residue by H/C correlation at δ 4.48/72.1 and 4.64/71.2, respectively.

Low-field shifts of the C2 resonance of Man[1], the C6 resonance of Man[2], the C3 resonance of GalNAc, and C4 resonance of GalNAcA to δ 77.1, 66.6, 76.4 and 77.2, respectively, in comparison with their positions in the unsubstituted monosaccharides at δ 71.7, 62.0, 72.0, and 70.4, respectively [15, 16], confirmed the substitution types of these monosaccharides determined by the methylation technique.

The α-configuration of the Man[1] and Man[2] glycoside bonds was confirmed according to the positions of C5 resonances at δ 74.4 and 73.2 [17].

The monosaccharide sequence in the repeating unit was determined by the $^{1}H,^{13}C$ HMBC experiment. The following interresidue correlations between the anomeric protons and the linkage carbon atoms were revealed: Man[1] H1/Man[2] C6, Man[2] H1/GalNAcA C4, GalNAcA H1/GalNAc C3, and GalNAc H1/Man[1] C2 at δ

4.91/66.6, 5.25/77.2, 5.16/76.4, and 4.51/77.1, respectively.

The polysaccharide was subjected to solvolysis to confirm the full structure of repeating unit and this provided additional information. The fragment obtained by this was analyzed by NMR spectroscopy as described above for the initial OPS. The ^{1}H and ^{13}C NMR spectra demonstrated that this fragment is the disaccharide consisting of GalNAcA and GalNAc residues. The last residue is on the reducing end and substituted in position 3.

To ascertain the relative absolute configurations of Gal*p*NAc and Gal*p*NAcA, glycosylation effects [17, 18] in the ^{13}C NMR spectra of the polysaccharide and the disaccharide (table) were examined. A small positive β-effect of 0.1 ppm for C-3 of D-Man*p*^{I} in the ^{13}C NMR spectrum of the OPS showed that Gal*p*NAc and D-Man^I in a β(1→2)-linked disaccharide have the same absolute configurations (a higher by absolute value negative β-effect would be expected if the absolute configurations were different). Indeed, a relatively small α-effects of 4.6 ppm for C-1 of Gal*p*NAc and 5.1 ppm for C-2 of D-Man*p* also indicated the same D-configuration (higher α-effects of ~8 ppm for C-1 of Gal*p*NAc and for C-2 of D-Man*p* would be observed in case of their different configurations). A small negative β-effect of –0.2 and –0.1 ppm for C-4 of α-D-Gal*p*NAc and β-D-Gal*p*NAc, respectively, in the ^{13}C NMR spectrum of α-Gal*p*NAcA-(1→3)-D-Gal*p*NAc disaccharide and also large value α-effect of about 8 ppm for C-1 of α-Gal*p*NAcA indicated different absolute configurations of Gal*p*NAcA and Gal*p*NAc (on the contrary, a higher absolute value negative β-effect of ~ –4 ppm for C-4 of D-Gal*p*NAc and relatively small α-effect of about 3 ppm for C-1 of α-Gal*p*NAcA would show the same absolute configurations).

The negative optical rotation of Gal*p*NAcA, $[\alpha]_D^{20}$ –16°, specific according to Klyne's rule on the basis of the optical rotation of the disaccharide obtained by solvolysis, $[\alpha]_D^{20}$ –40°, is additional proof of the L-configuration of Gal*p*NAcA. According to published data, the specific optical rotation of D-Gal*p*NAcA is $[\alpha]_D^{20}$ +29° [19].

On the basis of our results, the O-specific polysaccharide from *A. palladensis* KMM 3961^T has the following structure:

$$\underset{\text{A}}{\rightarrow 2)\text{-}\alpha\text{-D-Man}p^1\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p^2\text{-}(1\rightarrow 4)\text{-}}$$
$$\underset{\text{B}}{}$$

$$\underset{\text{C}}{\text{-}\alpha\text{-L-Gal}p\text{NAcA-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow}$$
$$\underset{\text{D}}{}$$

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