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Structure of an Acidic O-Specific Polysaccharide of the Marine Bacterium *Pseudoalteromonas agarivorans* KMM 232 (R-form)

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Abstract—An acidic O-specific polysaccharide containing L-rhamnose, 2-acetamido-2-deoxy-D-galactose, 2,6-dideoxy-2-(N-acetyl-L-threonine)amino-D-galactose, and 2-acetamido-2-deoxy-D-mannuronic acid was obtained by mild acid degradation of the lipopolysaccharide of the marine bacterium *Pseudoalteromonas agarivorans* KMM 232 (R-form) followed by gel-permeation chromatography. The polysaccharide was subjected to Smith degradation to give a modified polysaccharide with trisaccharide repeating unit containing L-threonine. The initial and modified polysaccharides were studied by sugar analysis and ^{1}H- and ^{13}C-NMR spectroscopy, including COSY, TOCSY, ROESY, and HSQC experiments, and the structure of the branched tetrasaccharide repeating unit of the polysaccharide was established.

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Key words: *Pseudoalteromonas agarivorans*, marine bacteria, bacterial polysaccharide structure, O-specific polysaccharide, NMR spectroscopy, threonine

Microorganisms of the marine environment are an important component of marine ecosystems and possess a wide spectrum of physiological adaptive mechanisms. Marine bacteria have very diverse habitats including coastal and open water areas, deep-sea and hydrothermal vents, and polar environments. Microorganisms are able to colonize external envelops and internal tissues of marine animals and plants; certain microbial groups can form highly specific symbiotic relationships with host organisms.

Gram-negative, aerobic, non-fermentative, heterotrophic, and halophilic bacteria of the genus *Pseudoalteromonas* can be considered to be obligate marine microorganisms. They are widespread in marine environments and produce a great number of biologically active substances [1]. The genus *Pseudoalteromonas* now contains about 40 validly described species [2]. Most species are representatives of bacteria associated with marine biota.

In recent years extensive studies of polysaccharides produced by *Pseudoalteromonas* spp. have revealed common features of most of them – acidic character and the presence of non-sugar substituents [3, 4].

On the basis of physiological, biochemical, and genetic properties, agarolytic strain KMM 232 was preliminary identified as *Pseudoalteromonas marinoglutinosa*. But further phenotypic, phylogenetic, and DNA–DNA relatedness studies showed that bacterium KMM 232 is a novel species of the genus *Pseudoalteromonas*, which was named *P. agarivorans* [5].

A distinctive feature of the agarolytic strain KMM 232 is that it forms R-type (rough) colonies (Fig. 1a) together with S-type (smooth) colonies (Fig. 1b) during its cultivation on solid media. The R-form of KMM 232 has traits that are typical characteristics of R-bacteria, including R-colony formation, loss of flagella, and high sensitivity to antibiotics. The dissociation of R- and S-forms was observed to be stable for the strain KMM 232 and in other strains of the species *P. agarivorans*. This was the first time R- and S-form dissociation was described for bacteria of the genus *Pseudoalteromonas*.

Earlier we established the structure of O-specific polysaccharide from lipopolysaccharide of *P. marinogluti-*

Abbreviations: COSY, correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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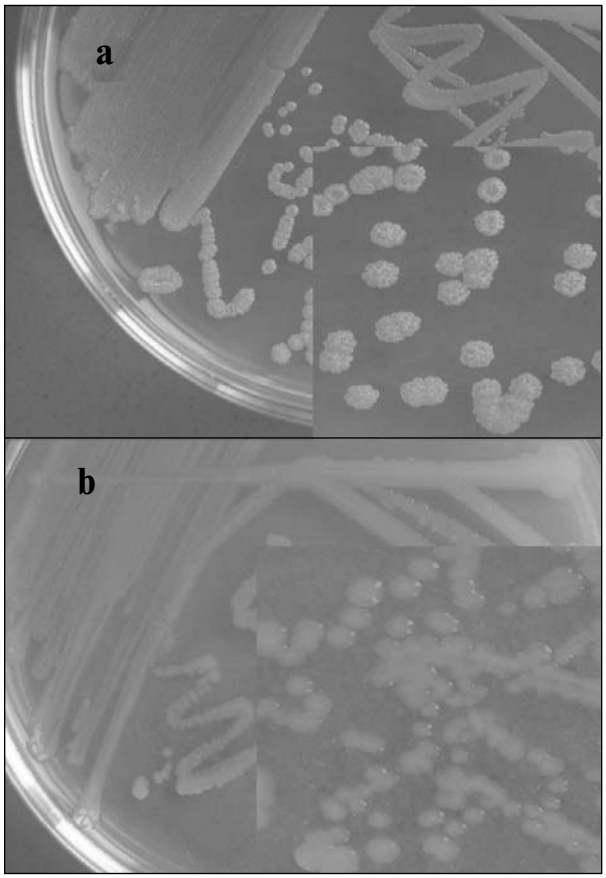

Fig. 1. Forms of colonies of bacterium *Pseudoalteromonas agarivorans* KMM 232: a) R-form; b) S-form.

nosa KMM 232 (S-form) that consists of disaccharide repeating units containing a sulfate group [6].

This paper presents the results of structural investigation of R-form of the marine proteobacterium *P. agarivorans* KMM 232.

MATERIALS AND METHODS

The microorganism *P. agarivorans* KMM 232 (R-form) was isolated from a seawater sample taken at a depth of 500 m in the northwest area of the Pacific Ocean. This strain was taken from the Collection of Marine Microorganisms of the Pacific Institute of Bioorganic Chemistry. The bacterium was grown for 3 days on solid medium that contained: 5 g peptone, 2.5 g yeast extract, 1 g glucose, 1 g K_2HPO_4, 0.05 g $MgSO_4$, 750 ml marine water, 250 ml distilled water, and 18 g agar.

The lipopolysaccharide was isolated from dry bacterial cells by extraction with hot aqueous phenol [7]. Nucleic acids and proteins were removed by precipitation with trichloroacetic acid at pH 2.0. The precipitate was removed by centrifugation, and the supernatant was dialyzed against distilled water and lyophilized. The lipopolysaccharide was isolated with a yield of about 10%.

The lipopolysaccharide was degraded with aqueous 1% acetic acid for 3 h at 100°C, and precipitate of lipid A was removed by centrifugation (13,000*g*, 20 min). The supernatant was fractionated by gel-permeation chromatography on column (100 × 1.5 cm) with TSK-HW-50 (S) (Merck, Germany) in 0.3% acetic acid. Elution curves were plotted with a RIDK 101 differential refractometer (Czechia). As a result, the O-specific polysaccharide was obtained with a yield of about 19%.

Periodate oxidation of the O-specific polysaccharide (30 mg) was performed in 0.1 M solution of sodium metaperiodate (2 ml) for 3 days at 25°C in darkness. Sodium borohydride (50 mg) was added, and after 4 h the excess of sodium borohydride was destroyed by acetic acid. The reaction mixture was dialyzed against distilled water and evaporated *in vacuo*. The product was heated with 1% acetic acid (2 ml, 2 h at 100°C), and the modified polysaccharide (9 mg) was isolated by gel-filtration chromatography on a column (80 × 1 cm) with TSK-HW-40 (S) (Merck) in water.

The polysaccharide was subjected to complete acid hydrolysis with 2 M hydrochloric acid for 3 h at 100°C. Monosaccharides were analyzed by paper chromatography and gas–liquid chromatography (GLC) as polyol acetates. Descending analytical and preparative chromatographies were performed on Filtrak FN-12 paper in butan-1-ol–pyridine–water (6 : 4 : 3 v/v); neutral monosaccharides were detected with alkaline solution of $AgNO_3$ and amino sugars with 0.2% solution of ninhydrin in acetone.

GLC was performed using an Agilent 6850 chromatograph (USA) equipped with a capillary column of 5MS 5% phenylmethylsiloxane stationary phase over the temperature range 150 to 230°C at 3°C/min. For amino sugar identification the polysaccharide was hydrolyzed with 4 M HCl (4 h at 100°C). After evaporation, the hydrolyzate was studied using an LKB Biochrom 4151 Alpha Plus amino acid analyzer (Sweden) on a column (200 × 4.6 mm) with Ultra Pak (8 μ) resin using 0.2 M sodium citrate buffer (pH 6.46) at 50-85°C. Uronic acid was identified using GLC and GLC–mass spectrometry of the acetylated methyl glycoside, which was prepared by methanolysis of the polysaccharide with 1 M HCl in methanol (100°C, 3 h) followed by acetylation. GLC–mass spectrometry was performed on Hewlett Packard 5890 chromatograph (USA) equipped with a capillary column of 5MS 5% phenylmethylsiloxane stationary phase and connected with a Hewlett Packard 5973 mass spectrometer (USA) for the temperature range 120-225°C at 3°C/min.

The absolute configuration of residues of rhamnose and threonine was determined by measuring their specific optical rotations on a Perkin-Elmer 141 instrument.

Free monosaccharide and amino acid were isolated from polysaccharide hydrolyzate using descending preparative paper chromatography.

The isolated L-rhamnose, $[\alpha]_{578}$ +6° (0.2 g per 100 ml water) was treated with 1 M HCl in 2 ml methanol for 2 h at 100°C and thus converted into methyl-L-rhamnopyranoside, $[\alpha]_{578}$ −65.4° (0.1 g per 100 ml water); from the literature data α-methyl-L-rhamnopyranoside has $[\alpha]_{578}$ −67.2°, water [8].

The specific optical rotation of threonine residue $[\alpha]_D^{20}$ −15° (0.5 g per 100 ml water) confirmed its L-configuration; in the literature data the specific optical rotation for L-threonine is $[\alpha]_D^{20}$ −27.4° (1 g per 100 ml water), D-threonine $[\alpha]_D^{20}$ +27° (1 g per 100 ml water) [9].

NMR spectra were run on a Bruker DRX-500 spectrometer (Germany) in D_2O solutions at 30°C using acetone (σ_C 31.45, σ_H 2.22) as the internal standard. Prior to measurement, samples were lyophilized from D_2O. Two-dimensional spectra were recorded using standard Bruker software; data were acquired and processed using the XWINNMR 2.1 program. Mixing times of 200 and 100 msec were used in TOCSY and ROESY experiments, respectively.

RESULTS AND DISCUSSION

The O-specific polysaccharide of *P. agarivorans* KMM 232 (R-form) was obtained by mild acid degradation of the lipopolysaccharide isolated from the bacterial cells by hot phenol–water extraction [7] followed by gel chromatography. It was not possible to isolate lipopolysaccharide from bacterial cells by the method of Galanos [10] specific to R-forms of bacteria, indicating the presence in lipopolysaccharide of O-specific polysaccharide. GLC and GLC–mass spectrometric analysis of the acetylated polyols and methyl glycosides revealed residues of rhamnose (Rha), fucosamine (FucN), galactosamine (GalN), and hexosaminuronic acid. Amino acid analysis confirmed the presence of FucN and GalN and showed the presence of threonine (Thr). The absolute (L) configuration of the rhamnose and threonine residues was defined on the basis of their specific optical rotation. The absolute configuration of the other monosaccharides and exact identification of hexosaminuronic acid was established by NMR spectroscopy, including one- and two-dimensional experiments.

The ^{13}C-NMR spectrum of the O-specific polysaccharide (Fig. 2 and the table) contained signals for four anomeric carbons at δ 93.7, 96.0, 99.4, and 103.5 ppm; three CH_3-C groups at δ 16.5, 17.9, and 18.2 ppm (C6 of a 6-deoxysugar and C4 of Thr, respectively), three carbons bearing nitrogen at δ 48.8, 49.0, and 54.2 ppm (C2 of amino sugars), C2 atom of Thr at 60.4 ppm, carbon of hydroxymethyl group at 63.1 ppm (C6 of GalN), other sugar ring carbons linked to oxygen in the region δ 68.6-78.2 ppm, carbonyl groups at δ 172.0-175.8 ppm, and three N-acetyl groups at δ 23.2-23.3 ppm. The absence from signals of non-anomeric sugar carbons at lower field than δ 81 ppm in the ^{13}C-NMR spectrum demonstrated the pyranoid form of all sugar residues [11].

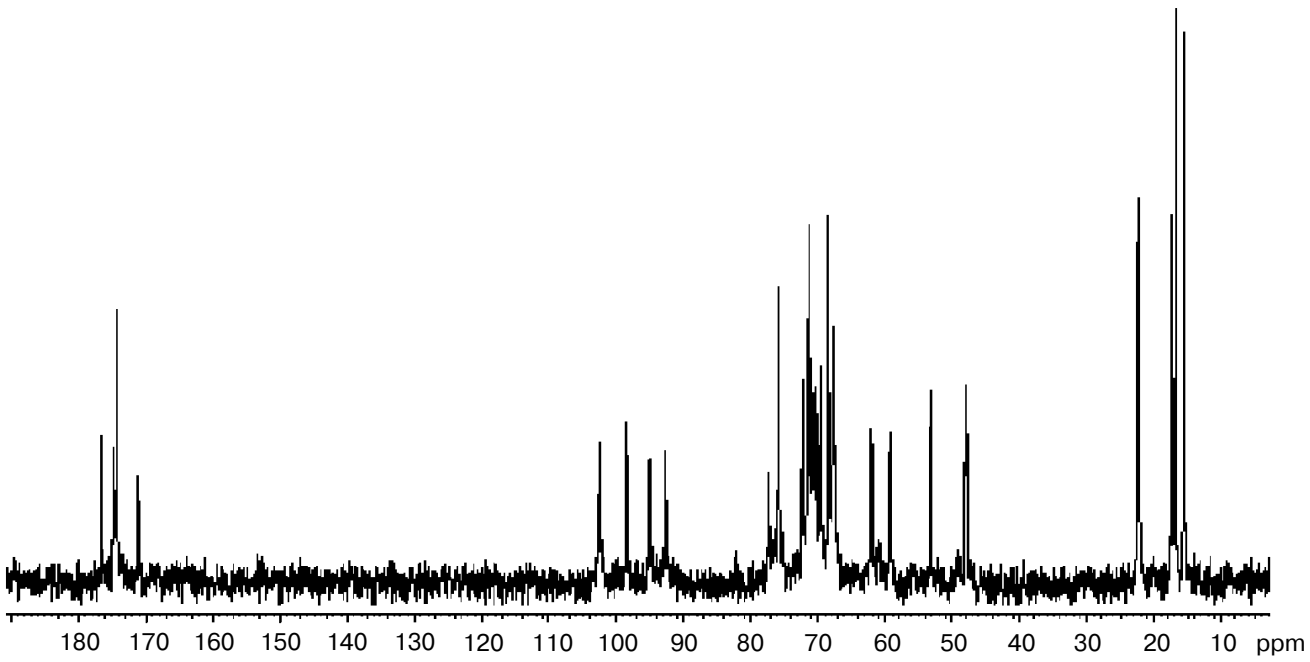

Fig. 2. ^{13}C-NMR spectrum of the O-specific polysaccharide of *P. agarivorans* KMM 232.

Data of the ^{1}H- and ^{13}C-NMR spectra of the initial and modified polysaccharides of *P. agarivorans* KMM 232 (δ, ppm)

Sugar residue	H/C	1	2	3	4	5	6 (6a,6b)
O-Specific polysaccharide*							
→3)-α-D-Fuc*p*N-(1→	^{1}H	5.18	4.43	3.65	3.21	3.92	1.26
	^{13}C	93.7	48.8	78.2	69.5	68.7	16.5
→3,4)-α-D-Gal*p*NAc-(1→	^{1}H	5.16	4.42	4.06	4.68	3.86	3.82; 3.58
	^{13}C	96.0	49.0	71.6	72.4	68.6	63.1
→3)-α-L-Rha*p*-(1→	^{1}H	4.82	3.82	3.93	3.51	3.78	1.26
	^{13}C	103.5	72.0	76.8	71.5	71.2	17.9
β-D-Man*p*NAcA-(1→	^{1}H	4.55	4.60	3.55	3.80	3.25	
	^{13}C	99.4	54.2	73.3	70.5	76.8	177.6
L-ThrAc	^{1}H		4.53	4.29	1.21		
	^{13}C		60.4	69.5	18.2		
Modified polysaccharide**							
→3)-α-D-Fuc*p*N-(1→	^{1}H	5.05	4.46	3.90	4.23	3.96	1.26
	^{13}C	95.5	48.7	77.7	69.3	68.8	16.5
→3)-α-D-Gal*p*NAc-(1→	^{1}H	5.18	4.34	4.06	4.47	3.86	3.83; 3.80
	^{13}C	96.4	49.2	73.7	66.7	71.0	62.1
→3)-α-L-Rha*p*-(1→	^{1}H	4.90	3.96	3.97	3.51	3.78	1.27
	^{13}C	103.1	78.0	77.1	71.4	70.7	17.8
L-ThrAc	^{1}H		4.44	4.24	1.23		
	^{13}C		60.2	69.3	18.9		

* Chemical shifts for *N*-acetyl groups are: δ_H 2.05-2.18; δ_C 23.2-23.3 (Me) and 172.0-175.8 (CO).
** Chemical shifts for *N*-acetyl groups are δ_H 2.03; 2.06; δ_C 23.2 (2 Me) and 171.6-175.5 (CO).

In the gated-decoupling ^{13}C-NMR spectrum the signals for C1 at 93.7, 96.0, and 103.5 ppm displayed a $^{1}J_{C1\text{-}H1}$ coupling constant of 170 Hz and, hence, belonged to α-linked sugars. The fourth C1 signal at 99.4 ppm was characterized by lower $^{1}J_{C1\text{-}H1}$ coupling constant value of 163 Hz, thus indicating that the remaining monosaccharide is β-linked [12].

Correspondingly, the ^{1}H-NMR spectrum contained signals for four anomeric protons at δ 4.55-5.18 ppm, methyl groups (H4 of Thr and H6 of 6-deoxy sugars) at δ 1.21 and 1.26 ppm (double integral intensive), and also N-acetyl groups at δ 2.05-2.18 ppm.

Thus, the polysaccharide consists of tetrasaccharide repeating units containing one residue each of L-Rha, FucN, GalN, L-Thr, and hexosaminuronic acid.

For further structural analysis, ^{1}H- and ^{13}C-NMR spectra were assigned using 2D homonuclear COSY, TOCSY, ROESY, and heteronuclear $^{1}H,^{13}C$ HSQC experiments.

Spin systems of GalN and FucN were identified based on correlations for all ring protons found in the COSY and TOCSY spectra. Configurations of α-GalN and α-FucN residues were confirmed by correlation between protons at the nitrogen-linked carbons with the corresponding carbons at δ 4.42/49.0 (H2/C2) and 4.43/48.8 (H2/C2), respectively.

The COSY and TOCSY spectra showed cross peaks of H1-H2 and of H2 up to H6 for the α-Rha residue.

The last monosaccharide was assigned as 2-acetamido-2-deoxy-mannuronic acid (ManNAcA) based on correlations between protons of H1-H2 and of H2 up to H5. Besides, relatively small $^{3}J_{H1,H2}$ coupling constant (~2 Hz) determined from the ^{1}H-NMR spectrum, H2/C2 cross peaks at δ 4.60/54.2 (found in the HSQC experiment) are characteristic for 2-amino-2-deoxymannuronic acids. Comparison of chemical shifts for H5 and C5 with published data [13] suggests that ManNAcA is β-linked.

Signals of anomeric protons and the corresponding carbons at δ 5.18/93.7, 5.16/96.0, 4.82/103.5, and 4.55/99.4 were assigned from $^{1}H,^{13}C$ HSQC spectra for FucN, GalN, Rha, and ManNA, respectively. A fragment of the two-dimensional $^{1}H,^{13}C$-HSQC spectrum of the O-specific polysaccharide is given in Fig. 3.

The correlations defined for signal of the CH_3-group at δ 1.21 ppm in COSY and TOCSY spectra corresponded to the amino acid Thr.

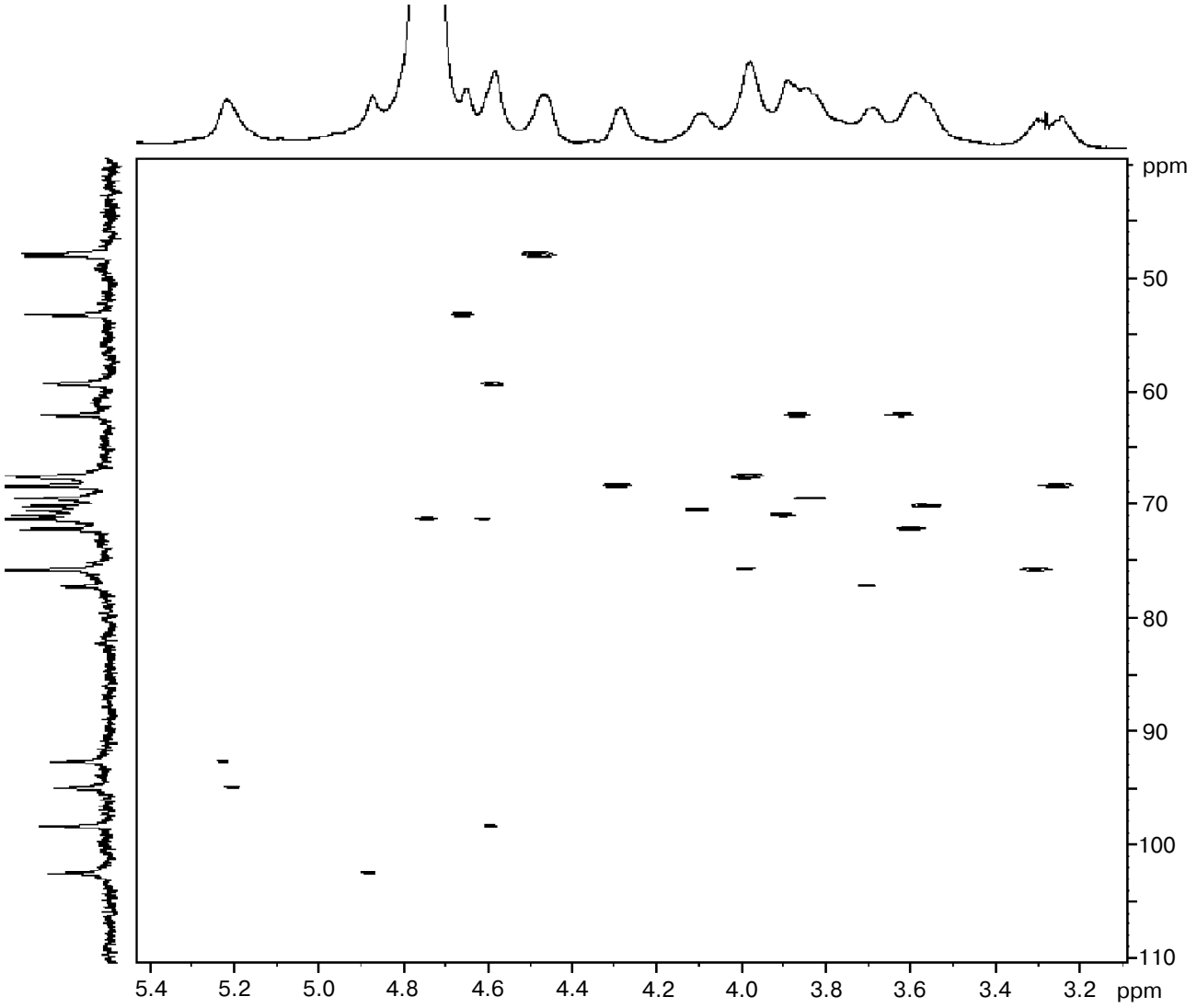

Fig. 3. Fragment of two-dimensional $^{1}H,^{13}C$-HSQC spectrum of O-specific polysaccharide of *P. agarivorans* KMM 232.

To confirm the full structure of the repeating unit, the polysaccharide was subjected to Smith degradation. The resulting modified polysaccharide was studied by 1D and 2D NMR spectroscopy as described above for the initial polysaccharide. It was found that the modified polysaccharide contained α-FucN, α-GalN, α-Rha, and Thr. Analysis of ^{1}H- and ^{13}C-NMR spectra of the modified polysaccharide was performed using 2D COSY, TOCSY, ROESY, and $^{1}H,^{13}C$ HSQC experiments, including the full assignment of ^{1}H- and ^{13}C-NMR signals (table).

Comparison of the ^{13}C-NMR spectra for the initial and modified polysaccharide showed that the characteristic signals at δ 99.4, 54.2, and 177.6 ppm for β-Man*p*NAcA in ^{13}C-NMR spectrum were absent in the modified polysaccharide. This indicated oxidation of the given monosaccharide residue and its location in a side branch.

Linear character of the modified polysaccharide followed from relatively low-field positions of the signals for C3 FucN, GalN, and Rha at δ 77.7, 73.7, and 77.1 ppm, respectively. A 2D ROESY experiment revealed strong inter-residue cross peaks between the anomeric protons and protons at the linkage carbons at δ 5.05/4.06, 5.18/3.97, and 4.90/3.90 ppm, which were assigned to H1 α-Fuc*p*N/H3 α-GalN, H1 α-GalN/H3 α-Rha, and H1 α-Rha/H3 α-Fuc*p*N correlations, respectively.

The position of the threonine residue was determined from correlation between the proton at C2 of Thr and the proton at C1 of Rha. Besides, the ^{13}C-NMR spectrum of the modified polysaccharide contains signal of an N-acetyl group at δ 23.2 (double integrated intensity), which indicates that all amino groups (one of which belongs Thr) are N-acetylated. Hence Thr is N-acetylated.

The absolute configuration of FucN and GalN was ascertained on the basis of the values of chemical shifts and of the ^{13}C-glycosylation effects, and the monosaccharides were found to have D-configuration [14].

On the basis of these data, the modified polysaccharide has the following structure:

→3)-α-D-Fuc*p*NThrAc-(1→3)-α-D-Gal*p*NAc-(1→3)-α-L-Rha*p*(1→.

The absolute configuration of residue Man*p*NAcA was not specially defined, and in this article it is accepted that this monosaccharide has the same D-configuration as in all other natural carbohydrates determined earlier [15-17].

Comparing the HSQC spectrum of the initial and modified polysaccharides, the H4/C4 cross-peak of Gal*p*NAc in the modified polysaccharide shifted upfield from δ 4.68/72.4 to 4.47/66.7. This displacement confirmed that Man*p*NAcA is linked at position 4 of Gal*p*NAc.

Thus, based on all our data, we suggest the following structure for the repeating unit of the O-specific polysaccharide of R-form *P. agarivorans* KMM 232:

β-D-Man*p*NAcA
1
↓
4
→3)-α-D-Fuc*p*NThrAc-(1→3)-α-D-Gal*p*NAc-(1→3)-α-L-Rha*p*-(1→.

To our knowledge, one of the polysaccharide components, namely, 2,6-dideoxy-2-(N-acetyl-L-threonine)amino-D-galactose, has not been found earlier in nature.

As is known, R-forms of terrestrial bacteria, unlike S-forms lose O-specific chains of the lipopolysaccharides and thus attain other properties. A feature of the marine bacterium *P. agarivorans* KMM 232 is that its S- and R-forms also synthesize lipopolysaccharides, in which O-specific polysaccharides have various structures.

In the given strain we do not observe loss of O-specific polysaccharides in the R-form and we consider that probably such variability reflects readiness of the bacterium to adapt to environmental changes. Moreover, it can show that Gram-negative marine bacteria substantially differ from terrestrial bacteria by structural organization and mechanism of functioning of cell wall.

On the basis of these results, we consider that the O-specific polysaccharide of marine bacteria not only causes specificity of a microorganism, but also has other functions.

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