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Structure of O-Specific Polysaccharide from *Pseudoalteromonas nigrifaciens* Strain KMM 161

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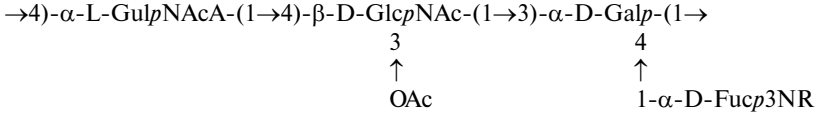
Abstract—On mild acid degradation of the lipopolysaccharide of the marine microorganism *Pseudoalteromonas nigrifaciens* KMM 161 an O-specific polysaccharide containing D-galactose, 2-acetamido-2-deoxy-D-glucose, 3,6-dideoxy-3-(4-hydroxybutyramido)-D-galactose, and 2-acetamido-2-deoxy-L-guluronic acid residues was obtained. From the results of Smith degradation, O-deacetylation of the polysaccharide, and NMR spectroscopy the following structure of the tetrasaccharide repeating unit of the O-specific polysaccharide was established:



where R: -CO-CH_2-CH_2-CH_2-OH. It should be noted that the same structure occurs in the antigenic polysaccharide of *Pseudoalteromonas nigrifaciens* KMM 158 described earlier as *Alteromonas macleodii* 2MM6.

Key words: *Pseudoalteromonas nigrifaciens*, O-specific polysaccharide, 4-hydroxybutanoic acid, NMR spectroscopy

Marine Proteobacteria are typical procaryotes that occupy various ecological niches and represent a significant part of the microbial community of the World Ocean. Bacteria of *Pseudoalteromonas* genus are marine aerobic Gram-negative microorganisms [1, 2]. They are of interest for their ability to synthesize a wide range of biologically active compounds such as antibiotics, toxins, antitoxins, anticancer and antimicrobial substances, and enzymes of wide spectrum of specificities [3]. At the same time, the composition and structure of the carbohydrate-containing biopolymers of the bacterial cell wall of pseudoalteromonads are studied rather poorly. Earlier, we and other authors established the primary structure of antigenic polysaccharides (PS) of several species of marine bacteria of the *Pseudoalteromonas* genus containing unusual acidic monosaccharides, N-acylamino sugars, and non-carbohydrate substituents [4-13].

The purpose of the present work is the structural analysis of the O-specific PS of the marine Proteobacterium *Pseudoalteromonas nigrifaciens* KMM 161.

MATERIALS AND METHODS

The strain *P. nigrifaciens* KMM 161 from the Collection of Marine Microorganisms of the Pacific Institute of Bioorganic Chemistry was isolated from mantle of sea mussel *Patinopecten yessoensis* (Troitsa bay, gulf of Peter the Great, Sea of Japan). The procedures of isolation, cultivation, primary identification, and storage of the strain were described earlier [14]. The strain was isolated from an individual colony after incubation for 7 days at 28°C on agar medium of the following composition (g/liter): peptone, 5.0; yeast extract, 2.0; glucose, 1.0; K_2HPO_4, 0.2; casein hydrolyzate, 2.0; $MgSO_4$, 0.05; agar, 20.0; distilled water, 500 ml; natural seawater, 500 ml (pH 7.8).

The microorganism was cultivated with shaking (160 rpm) for 36 h at room temperature in liquid medium (g/liter): peptone, 5.0; yeast extract, 2.0; glucose, 1.0;

Abbreviations: LPS) lipopolysaccharide; PS) polysaccharide; PC) paper chromatography; GLC) gas-liquid chromatography; GLC-MS) gas-liquid chromatography/mass-spectrometry.

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K_2HPO_4, 0.2; $MgSO_4$, 0.05; distilled water, 500 ml; natural seawater, 500 ml (pH 7.8). Bacterial cells were isolated by centrifugation at 5000 rpm.

^{13}C-NMR spectra were recorded on a Bruker WM-250 spectrometer with solutions in D_2O at 60°C (methanol as internal standard, δ_C 50.15 ppm).

Solutions were freeze-dried or evaporated *in vacuo* at 40°C. Optical rotation was measured on a Perkin-Elmer 141 polarimeter for solutions in water at 20°C. Descending paper chromatography (PC) was carried out on Filtrak FN-15 and Whatman 3MM papers in *n*-butanol–pyridine–water (6 : 4 : 3), high-voltage paper electrophoresis in 0.025 M pyridine-acetate buffer (pH 4.5; 30 V/cm) with detection of monosaccharides by alkaline silver nitrate. Gel chromatography was performed on a column (2.5 × 100 cm) with TSK HW 50 (F) gel in 0.3% acetic acid with monitoring by RIDK 101 (Czechia) differential refractive index detector. Gas-liquid chromatography (GLC) was performed on a Pye Unicam 104 instrument equipped with a glass column (0.4 × 150 cm) packed with 3% QF-1 on a Gas Chrom Q within the temperature range of 180→225°C. GLC-MS was carried out on an LKB 9000S mass-spectrometer. Amino acid analysis was performed on the Biotronik LC-2000 instrument on a column (0.22 × 6 cm) with DC 6A resin.

The strain of a marine microorganism *P. nigrifaciens* KMM 161 was used in this work. Isolation of lipopolysaccharide (LPS) was carried out as described earlier [4].

Isolation of the O-specific polysaccharide (PS). LPS (300 mg) was hydrolyzed with 1% acetic acid (30 ml, 100°C, 3 h), lipid A was removed by centrifugation (20 mg, 6.7% w/w), the supernatant was concentrated, and the O-specific PS was obtained by size-exclusive chromatography on TSK HW 50 (F) gel (Toyo Soda, Japan) (240 mg, 80% w/w).

Complete acid hydrolysis of PS (2 mg) was performed with 2 M CF_3COOH (0.5 ml, 120°C, 2 h), the sugars obtained were analyzed by PC and GLC as corresponding polyol acetates. In preparative scale, 20 mg of PS and 2 ml of acid were used to obtain: D-glucosamine hydrochloride (4 mg), $[\alpha]_D$ +6.8° (C 0.4, water); D-galactose (3 mg), $[\alpha]_D$ +76.4° (C 0.3, water) (cf. with data [15]: $[\alpha]_D$ +8° and +75°, respectively); and 3,6-dideoxy-3-amino-D-galactose (5 mg), $[\alpha]_D$ + 24.2° (C 0.5, water) (cf. with data [16]: $[\alpha]_D$ +23.9° (water)). For amino acid analysis PS was hydrolyzed with 4 M HCl (100°C, 4 h).

Partial acid hydrolysis of PS (150 mg) was performed with 1% acetic acid (20 ml, 100°C, 2 h), hydrolyzate was concentrated and subjected to size-exclusion chromatography on TSK HW 50 (F) gel. The high-molecular-mass fraction (110 mg) and low-molecular-mass (20 mg) fraction were obtained. From the latter, 3,6-dideoxy-3-(4-hydroxybutyramido)-D-galactose (4 mg), $[\alpha]_D$ + 94.2° (C 1.0, water) (cf. with data [5]: $[\alpha]_D$ + 91°) was isolated by preparative PC.

Methanolysis of PS was carried out with 1 M HCl in absolute methanol (0.5 ml, 120°C, 3 h), methanolyzate was evaporated to dryness, acetylated, and analyzed by GLC-MS.

O-Deacetylation of PS (90 mg) was performed in 10 ml 50% aqueous ammonia (60°C, 3 h), and the reaction mixture was concentrated and desalted on TSK HW-40 (F) gel. The yield of modified polysaccharide was 60 mg.

RESULTS AND DISCUSSION

Lipopolysaccharide (LPS) was obtained by the Westphal procedure [17], and nucleic acids were removed by precipitation with trichloroacetic acid at pH 2; then the LPS was fractionated into the polysaccharide and lipid components by the mild acid hydrolysis. In this case, the lipid A content in LPS was only 6.7%. Additionally, PS was purified by size-exclusive chromatography on TSK-50 (F) gel.

Sugar analysis of PS by PC, GLC, and high-voltage paper electrophoresis showed the presence of D-galactose, 2-acetamido-2-deoxy-D-glucose, 3-acetamido-3,6-dideoxy-D-galactose (3-aminofucose), and an unidentified acidic sugar giving positive reaction with ninhydrin. The D-configuration of the indicated sugars was established on the basis of their specific optical rotation values. The amino sugar analysis at comparison with reference amino sugars by an amino acid analyzer confirmed the presence of glucosamine and 3-aminofucose in equimolar quantities as constituents of the PS. Besides, as a result of methanolysis of the PS, acetylating the sugars obtained, and further analysis of the mixture by GLC-MS with computer-assisted calculation of mass-spectra, acetylated methylglycosides of hexose, 2-aminohexose, and 3,6-dideoxy-3-aminohexose were identified.

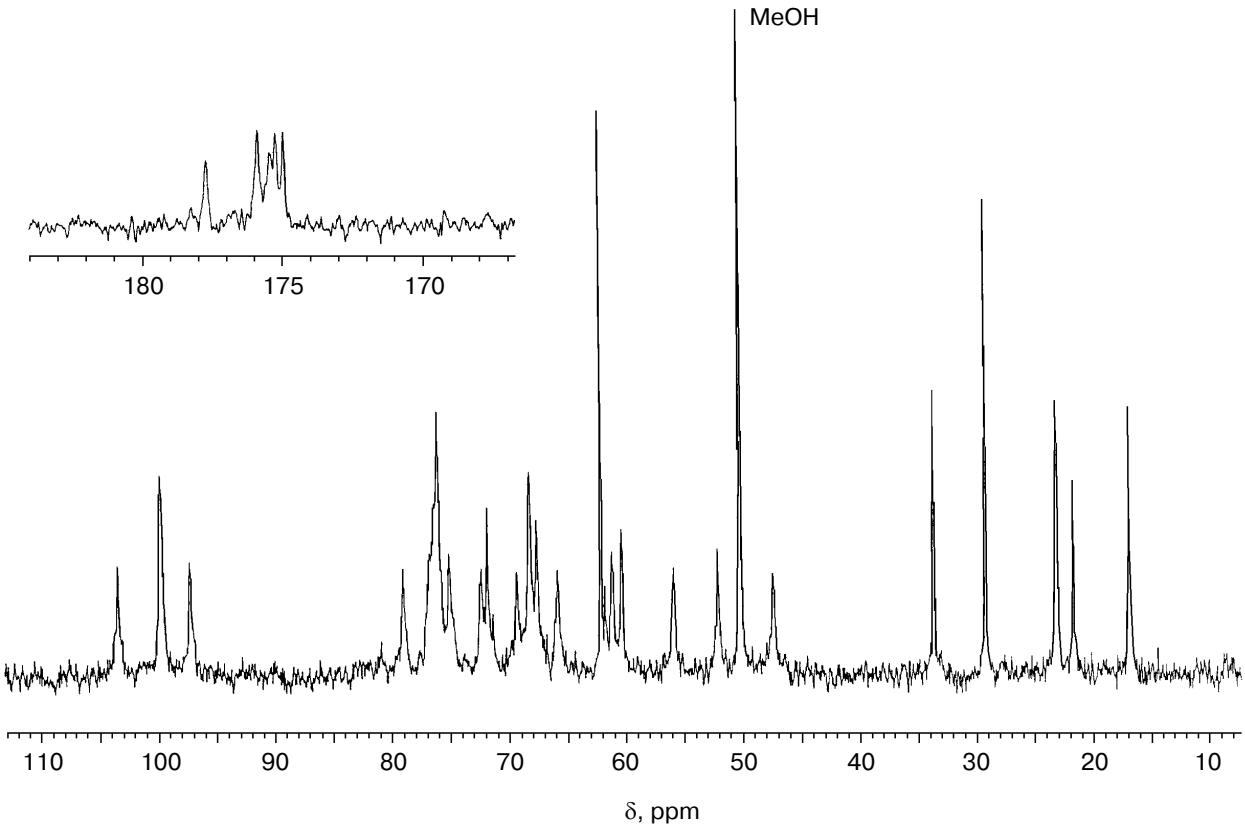
The ^{13}C-NMR spectrum of the PS (figure, table) indicated regular character and tetrasaccharide repeating unit (signals of four anomeric carbons at 97.2; 99.8 (double integral intensity), and 103.4 ppm). In the high-field the signals of a 6-deoxy group of 3-aminofucose at 16.8 ppm, O-acetyl group at 21.6 ppm, two N-acetyl groups (CH_3) at 23.0 and 23.1 ppm are observed. From spectral data obtained by us earlier for antigenic PS from *Alteromonas macleodii* 2MM6 [5] (now this microorganism is reclassified as *Pseudoalteromonas nigrifaciens* KMM 158 [14]), signals at 29.1, 33.6, 62.0, and 175.2 ppm contained in the spectrum are referred to 4-hydroxybutanoic acid residue acylating on the amino group of 3-aminofucose. In the low-field there are five signals of carbonyl carbons (C=O) in the region of 175-178 ppm; four of them belong to two acetamide, O-acetyl, and 4-hydroxybutyryl groups. Existence of the fifth low-field signal and also signals at 47.4 and 23.1 ppm indicate the presence of an amino uronic acid residue in the repeating unit. Moreover, signal C2 carrying N-acetyl group, in the high field (47.4 ppm) is characteristic for the only pyranose with α-*gulo*-configuration, that is easy to confirm, subtracting

^{13}C-NMR data of the O-specific polysaccharide from *P. nigrifaciens* KMM 161 (δ, ppm)*

Sugar residue	C1	C2	C3	C4	C5	C6
→4)-α-L-Gul*p*NAcA-(1→	99.8	47.7	65.8	75.0	68.2	177.7
	99.8	47.3	66.0	75.5	68.3	177.7
	(99.7)**	(47.4)	(65.8)	(75.0)	(68.2)	(177.7)
→3,4)-β-D-Glc*p*NAc-(1→	103.4	55.8	76.0	77.0	76.0	61.1
	104.4	57.4	74.2	76.4	76.0	61.3
	(103.2)	(55.7)	(76.3)	(77.2)	(76.0)	(61.5)
→3,4)-α-D-Gal*p*-(1→	97.3	69.3	79.0	76.0	72.3	60.0
	97.4	69.4	79.0	76.4	71.9	60.4
	(97.5)	(69.1)	(78.8)	(76.3)	(72.6)	(60.5)
α-D-Fuc*p*3N-(1→	99.8	67.6	52.0	71.8	68.2	16.8
	99.8	67.7	52.1	71.9	68.3	16.9
	(99.7)	(69.7)	(52.0)	(71.9)	(68.2)	(16.7)
-CO-CH_2-CH_2-CH_2-OH	174.9	33.6	29.1	62.0		
	175.0	33.6	29.2	62.1		
	(175.2)	(33.6)	(29.0)	(62.1)		

* In the second line in each column the data for the O-deacetylated PS are present.

** The data from [5] are present in the brackets. An additional signals of N- and O-acetyl groups are at 21.6, 23.0, 23.1 ppm (CH_3) and 174.9, 175.9, 177.7 ppm (C = O).



^{13}C-NMR spectrum of the O-specific polysaccharide from *P. nigrifaciens* KMM 161

usual (16-18 ppm) effect of a replacement of OH-group on NHAc from the magnitude of the chemical shift of the C2 in pyranoses [18].

From O-deacetylation under the mild alkaline conditions following gel-permeation chromatography, O-deacetylated PS was obtained. Its ^{13}C-NMR spectrum differed from the spectrum of the native polymer (table). The spectrum of the modified polymer, in the contrast of the spectrum of the intact PS, showed the absence of the signals of O-acetyl group at 21.6 (CH_3) and 173.9 ppm (C=O) that confirmed the full O-deacetylation. Besides, displacement of the chemical shifts of signals of C3 and C4 of the GlcNAc residue to high field by 1.8 and 0.6 ppm, respectively, and the signal of C2 of the same residue to low field by 1.6 ppm showed the location of the O-acetyl group in position 3 of this sugar. Displacement of the anomeric carbon signal of this residue to low field by 1 ppm, can possibly be explained by conformational changes in the spacing structure of the polymer. The data from the table show full coincidence of the chemical shift values in the spectra of PSs from *P. nigrifaciens* KMM 158 and *P. nigrifaciens* KMM 161, confirming full identity of their structures. The structure of the latter polymer was established on the basis of data of one-dimensional homonuclear experiment with coherent transfer of magnetic field (HOHAHA) [19], one-dimensional (NOE) and rotating-frame (ROESY) experiments [20], and two-dimensional heteronuclear ^{13}C/^{1}H correlated spectroscopy [21]. All these data are presented in [5] in detail.

It is interesting to note that the repeating unit of PS contains two rare sugars, 3,6-dideoxy-3-amino-D-galactose acylated by 4-hydroxybutanoic acid (such N-acyl derivative was identified earlier only in antigenic PS from *Pseudoalteromonas nigrifaciens* KMM 158 [5]) and 2-acetamido-2-deoxy-L-guluronic acid identified earlier in capsular PS of *Vibrio parahaemolyticus* K15 [22] and *Neisseria meningitidis* of group I [23]. Besides, from the data obtained it was shown that two strains isolated from the mantle of Far Eastern bivalve mollusks *Crenomytilus grayanus* (KMM 158) and *Patinopecten yessoensis* (KMM 161) were identified as *Pseudoalteromonas nigrifaciens* on the basis of its physiological and biochemical properties and DNA–DNA hybridization data [14].

REFERENCES

1. Baumann, L., Baumann, P., Mandel, M., and Allen, R. D. (1972) *J. Bacteriol.*, **110**, 402-409.
2. Gauthier, G., Gauthier, M., and Christen, R. (1995) *Int. J. Syst. Bacteriol.*, **45**, 755-761.
3. Fenical, W. (1993) *Chem. Rev.*, **93**, 1673-1683.
4. Gorshkova, R. P., Nazarenko, E. L., Zubkov, V. A., Ivanova, E. P., Ovodov, Y. S., Shashkov, A. S., and Knirel, Y. A. (1993) *Bioorg. Khim.*, **19**, 327-336.
5. Nazarenko, E. L., Zubkov, V. A., Shashkov, A. S., Knirel, Y. A., Gorshkova, R. P., Ivanova, E. P., and Ovodov, Y. S. (1993) *Bioorg. Khim.*, **19**, 740-751.
6. Nazarenko, E. L., Gorshkova, R. P., Zubkov, V. A., Shashkov, A. S., Ivanova, E. P., and Ovodov, Y. S. (1993) *Bioorg. Khim.*, **19**, 733-739.
7. Zubkov, V. A., Nazarenko, E. L., Gorshkova, R. P., Ivanova, E. P., Shashkov, A. S., Knirel, Y. A., Paramonov, N. A., and Ovodov, Y. S. (1995) *Carbohydr. Res.*, **275**, 147-154.
8. Gorshkova, R. P., Nazarenko, E. L., Zubkov, V. A., Shashkov, A. S., Knirel, Y. A., Paramonov, N. A., Meshkov, S. V., and Ivanova, E. P. (1997) *Carbohydr. Res.*, **299**, 69-76.
9. Gorshkova, R. P., Nazarenko, E. L., Isakov, V. V., Zubkov, V. A., Gorshkova, N. M., Romanenko, L. A., and Ivanova, E. P. (1998) *Bioorg. Khim.*, **24**, 839-841.
10. Gorshkova, R. P., Nazarenko, E. L., Zubkov, V. A., Shashkov, A. S., Ivanova, E. P., and Gorshkova, N. M. (1998) *Carbohydr. Res.*, **313**, 61-64.
11. Haffinity, O. M., Shashkov, A. S., Senchenkova, S. N., Tomshich, S. V., Komandrova, N. A., Romanenko, L. A., Knirel, Y. A., and Savage, A. V. (1999) *Carbohydr. Res.*, **321**, 132-138.
12. Perepelov, A. V., Senchenkova, S. N., Shashkov, A. S., Komandrova, N. A., Tomshich, S. V., Shevchenko, L. S., Knirel, Y. A., and Kochetkov, N. K. (2000) *J. Chem. Soc., Perkin Trans. I*, 363-366.
13. Rodeaux, H., Talaga, P., Carlson, W., and Guezennec, J. (1998) *Carbohydr. Res.*, **312**, 53-59.
14. Ivanova, E. P., Kiprianova, E. A., Mikhailov, V. V., Levanova, G. F., Garagulya, A. D., Gorshkova, N. M., Yumoto, N., and Yoshikawa, S. (1996) *Int. J. Syst. Bacteriol.*, **46**, 223-228.
15. Gorshkova, R. P., Isakov, V. V., Nazarenko, E. L., Ovodov, Y. S., Guryanova, S. V., and Dmitriev, B. A. (1993) *Carbohydr. Res.*, **241**, 201-208.
16. Jansson, P.-E., Lindberg, B., Spellman, M., Hofstad, T., and Skaug, N. (1985) *Carbohydr. Res.*, **137**, 197-203.
17. Westphal, O., Luderitz, O., and Bister, F. (1952) *Z. Naturforsch., Teil B*, **7B**, 148-155.
18. Shashkov, A. S. (1983) *Izv. AN SSSR. Ser. Khim.*, **6**, 1328-1336.
19. Subramanian, S., and Bax, A. (1987) *J. Magn. Reson.*, **71**, 325-330.
20. Bothner-By, A. A., Stephen, R. L., Lee, J., Warren, C. D., and Jeanols, R. W. (1984) *J. Amer. Chem. Soc.*, **106**, 811-813.
21. Shashkov, A. S., Lipkind, G. M., Knirel, Y. A., and Kochetkov, N. K. (1988) *Magn. Reson. Chem.*, **26**, 735-747.
22. Torii, M., Sakakibara, K., and Kuroda, K. (1973) *Eur. J. Biochem.*, **37**, 401-405.
23. Michon, F., Brisson, J. R., Roy, S., Ashton, F. E., and Jennings, H. J. (1985) *Biochemistry*, **24**, 5592-5598.