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STRUCTURE OF THE REPEAT UNIT OF THE *Alteromonas addita* TYPE STRAIN KMM 3600^{T} *O*-SPECIFIC POLYSACCHARIDE

R. P. Gorshkova, V. V. Isakov, V. A. Denisenko,
E. L. Nazarenko,* E. P. Ivanova, and L. S. Shevchenko

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The O*-specific polysaccharide of* Alteromonas addita *type strain KMM 3600^{T} is constructed of trisaccharide repeat units containing L-rhamnose, D-glucose, and D-galactose. It was established that the* O*-specific polysaccharide consists of trisaccharide repeat units with the structure →3)-α-D-Galp-(1→3)-α-L-Rhap-(1→3)-α-D-Glcp-(1→ based on monosaccharide analysis, Smith degradation, PMR and ^{13}C NMR spectroscopy, and two-dimensional COSY, HSQC, and HMBC.*

Key words: *Alteromonas addita*, *O*-specific polysaccharide, NMR spectroscopy.

The genus *Alteromonas* has been described as an independent taxon with gram-negative marine aerobic gamma-proteobacteria with one polar flagellum [1]. This genus at present includes 14 validly described species. A strain assigned according to phenotypic, chemotaxonomic, genetic, and phylogenetic features to the new species *A. addita* was isolated during taxonomic research of wild microbes from populations in Chasma Bay (Sea of Japan) [2, 3]. We have previously determined the structure of a lipooligosaccharide from *A. addita* strain KMM 3600^{T} [4]. Herein a structural investigation of the *O*-specific polysaccharide from *A. addita* of this same strain is reported.

A lipopolysaccharide (LPS) was isolated from wet bacterial cells of the marine proteobacterium *A. addita* by extraction with hot aqueous phenol and was purified of nucleic acids by precipitation with TCA at pH 2. Mild acid degradation of the LPS with subsequent anion-exchange and exclusion chromatography produced the *O*-specific polysaccharide (PS). Carbohydrate analysis of the PS using PC, GC, and GC—MS as the polyol acetates showed that the PS contained rhamnose, glucose, and galactose residues in a 1:1:1 ratio. All components of the PS were isolated pure by preparative PC. Glucose and galactose were assigned based on the specific optical rotations to the D-series; rhamnose, to the L-series.

The ^{13}C NMR spectrum (Fig. 1, Table 1) exhibited three resonances for anomeric C atoms at 103.1, 96.5, and 96.4 ppm; resonances of two hydroxymethyls at 62.2 and 62.1 ppm; a resonance for CH_3 of a 6-deoxysugar at 18.0 ppm; and 12 resonances of C ring atoms at 66-79 ppm. The spin—spin coupling constants (SSCC) for the anomeric C atoms that were determined from the ^{13}C NMR spectrum were recorded without suppression of C—H coupling (gated decoupling experiment) were $J_{C1,H1}$ = 170-171 Hz, which was consistent with the α-configuration of all monosaccharide residues [5]. The lack of resonances for non-anomeric C atoms at weak (>83 ppm) field in the ^{13}C NMR spectrum indicated that all monosaccharide residues had the pyranose form [6].

Smith degradation produced a polymer, the ^{13}C NMR spectrum of which was identical to that of the starting PS, which indicated that α-glycols were absent in the native PS. Therefore, all monosaccharide residues were α-1,3-bonded and the PS had a linear structure.

The PMR spectrum of the native PS contained resonances for three anomeric protons at 5.19 ppm (2H, $J_{H1\text{-}H2}$ = 3.1 Hz) and 5.10 ppm ($J_{H1\text{-}H2}$ = 1.2 Hz) in addition to a resonance for a CH_3–C group (H-6 of a 6-deoxysugar) at 1.31 ppm (J_{H6} = 6.2 Hz). The PMR and ^{13}C NMR spectra confirmed the PS structure as two hexose residues and one 6-deoxyhexose residue in the pyranose form bonded α-1,3 through glycoside bonds. This correlated well with the PS carbohydrate analysis.

Pacific Institute of Bioorganic Chemistry, Far-East Branch, Russian Academy of Sciences, 690022, Vladivostok, pr. 100-Letiya Vladivostoka, 159, fax (4232) 31 40 50, e-mail: elnaz@piboc.dvo.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 445-447, September-October, 2008. Original article submitted April 21, 2008.

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TABLE 1. NMR Spectra of *O*-Specific Polysaccharide from *Alteromonas addita* Strain 3600^T (δ, ppm)

Monosaccharide residue	C_1/H_1	C_2/H_2	C_3/H_3	C_4/H_4	C_5/H_5	C_6/H_6
→ 3)-α-L-Rha*p*-(1 →	103.1	68.0	76.3	71.6	70.4	18.0
	5.10	4.30	3.96	3.58	3.86	1.31
→3)-α-D-Glc*p*-(1 →	96.4	68.8	78.4	70.4	72.2	61.8
	5.19	3.98	4.04	3.77	4.05	3.78
→ 3)-α-D-Gal*p*-(1 →	96.5	67.8	75.4	66.7	71.7	62.4
	5.19	4.04	4.08	4.28	4.03	3.69

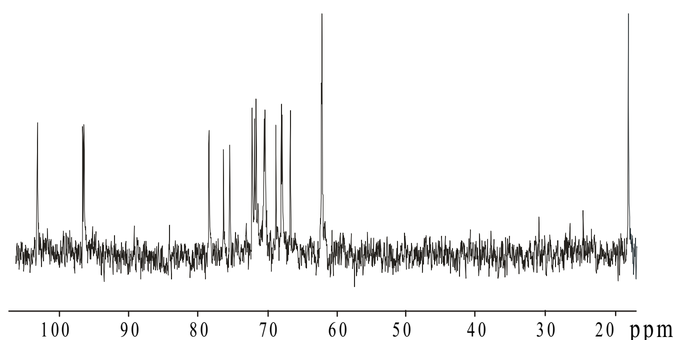

Fig. 1. ^{13}C NMR spectrum of *O*-specific polysaccharide from *Alteromonas addita* strain 3600^T (δ, ppm).

The two-dimensional (2D) COSY, HSQC, and HMBC experiments allowed resonances of C and H atoms to be assigned to the corresponding monosaccharide residues (Table 1). According to them, the C-3 resonances of all three monosaccharides at 76.3, 78.4, and 75.4 ppm were shifted to relatively weak field at 71.3, 74.0, and 70.1 ppm compared with their positions in the free monosaccharides (α-effect of glycosylation). This also was consistent with the presence of 1,3-glycosidic bonds.

Correlations were observed in the HMBC spectrum because of through-space inter-residue SSCC: H1 (δ 5.10) Rha/C3 (δ 78.4) Glc and H1 (δ 5.19) Glc/C3 (δ 75.4) Gal. This indicated that all monosaccharide residues were glycosylated in the 3-position with the following bonding sequence of monosaccharide residues in the PS: →3)-α-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→3)-α-D-Glc*p*-(1→.

The chemical shifts of the rhamnopyranose residue showed that the C-3 resonance at 76.3 ppm was shifted to weak field by +5.3 ppm (α-effect of glycosylation) whereas the C-2 resonance; to strong field by -4 ppm (β-effect of glycosylation) relative to their values in the unsubstituted rhamnose residue. Such values of the α- and β-effects of glycosylation are possible only for α-D→L absolute configurations of monosaccharide residues [5]. Furthermore, the chemical shifts of the anomeric C atoms agreed fully with the aforementioned sequence with respect to the glycosylation effects for 1,3-bonded monosaccharides [5].

Thus, the repeat unit of the *O*-specific PS from *A. addita* strain 3600^T had the structure →3)-α-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→3)-α-D-Glc*p*-(1→ according to the obtained results.

EXPERIMENTAL

The type strain *A. addita* KMM 3600^T from the Collection of Marine Microorganisms of PIBOC, FEB, RAS, was isolated from marine waters of Chasma Bay (Sea of Japan). The isolation, cultivation, preliminary identification, and preservation procedures have been published [3]. ^{13}C NMR spectra in D_2O were recorded on a Bruker DPX-300 instrument at 60°C with methanol internal standard (δ_C 50.15 ppm). PMR and 2D correlation spectra were recorded on a Bruker DRX-500 instrument with acetone internal standard (δ_H 2.225 ppm). Solutions were lyophilized or evaporated in vacuo. Specific optical rotation was measured on a Perkin—Elmer 141 polarimeter in water. Descending paper chromatography (PC) was performed

on Whatman 1 and Whatman 3MM paper using *n*-butanol:pyridine:water (6:4:3). Monosaccharides were detected using basic silver nitrate. Ion-exchange chromatography was performed on DEAE TSK 650M gel using tris-HCl buffer (0.5 M, pH 7.0). Gel chromatography was carried out over a column (2.5 × 90 cm) with TSK HW 40 (F) gel in water. Elution curves were constructed using an RIDK 101 (Czech Rep.) differential refractometer. LPS was isolated from wet microbial biomass by hot aqueous phenol (45%) using the Westphal method [7]. Nucleic acids were removed by precipitation with trichloroacetic acid (TCA) at pH 2. Yield of LPS was 50 mg from 1 L of medium.

Isolation of *O*-Specific Polysaccharide (PS). LPS (400 mg) was hydroyzed by CH_3COOH (1%, 40 mL, 100°C, 3 h). The precipitate of lipid A was removed by centrifugation (yield of lipid A, 20 mg). The aqueous phase was lyophilized and purified by anion-exchange chromatography over DEAE TSK 650M gel with subsequent gel chromatography over TSK HW 40 (F) gel. Yield of PS, 40 mg.

Smith Degradation. PS (20 mg) was dissolved in $NaIO_4$ (3 mL, 0.1 N), stored in the dark for 90 h, reduced with $NaBH_4$, neutralized by CH_3COOH, desalted by gel chromatography over TSK HW 40 (F) gel, and lyophilized. The polyalcohol was hydrolyzed by HOAc (1%, 1.5 h, 100°C) with subsequent gel chromatography over TSK HW 40 (F) gel and lyophilization. Yield of modified PS, 15 mg.

Hydrolysis of PS (2 mg) was carried out in CF_3COOH (2 M, 0.5 mL, 100°C, 3 h). The acid was removed by evaporation. The product was analyzed by PC, GC, and GC—MS as the polyol acetates. Preparative hydrolysis used PS (100 mg) and CF_3COOH (1 M, 5 mL, 100°C, 3 h). The hydrolysate was purified by preparative PC to afford D-glucose (14 mg), $[\alpha]_D$ +65° (lit. [8], +52.7°); D-galactose (13 mg), $[\alpha]_D$ +72° (lit. [8], +81.1°), and L-rhamnose (10 mg), $[\alpha]_D$ +6.0° (lit. [8], +9.1°). The last was converted to methyl-L-rhamnopyranoside (8 mg), $[\alpha]_D$ -65.5° (lit. [8], -62.5°), by methanolysis by an acetylchloride:methanol (1:10) mixture (1 mL, 10 h).

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