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O-Specific Polysaccharide of the Marine Bacterium "*Alteromonas marinoglutinosa*" NCIMB 1770

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Abstract—The O-specific polysaccharide of the marine bacterium "*Alteromonas marinoglutinosa*" NCIMB 1770 was obtained by mild acid degradation of the corresponding lipopolysaccharide and found to contain D-galactose, N-acetyl-D-glucosamine, and N-acetyl-D-mannosamine residues in equimolar ratio. Based on methylation analysis, periodate oxidation, and ^{13}C-NMR spectroscopy data of native and modified polysaccharides, the following structure of the trisaccharide repeating unit of the O-specific polysaccharide was established:

β-D-Man*p*NAc
1
↓
4
→3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→

Key words: "*Alteromonas marinoglutinosa*", marine bacteria, lipopolysaccharide, O-specific polysaccharide, structure, NMR spectroscopy

Polysaccharides of the cell wall of microorganisms play an important role in the functioning the bacterial cell and the interaction of bacteria with other biological systems, including the immune system of animals including humans. The large structural variety of O-specific polysaccharides and their important function in the ability of the microbial cell to survive make possible the application of these polymers in the systematics of gram-negative bacteria. Chemotyping and serotyping based on polysaccharides and lipopolysaccharides are widely used for classification of bacteria and elucidation of their phylogenetic relationship. However, the primary structures of O-specific polysaccharides of marine microorganisms are very poorly studied.

We established earlier the structure of the O-specific bacterial polysaccharide of *Pseudoalteromonas marinoglutinosa* KMM 232 from the Collection of Marine Microorganisms of the Pacific Institute of Bioorganic Chemistry, Far East Division of the Russian Academy of Sciences [1]. The task of the present research was to study the structure of the O-specific polysaccharide chain of the lipopolysaccharide of the marine bacterium "*Alteromonas marinoglutinosa*" NCIMB 1770.

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MATERIALS AND METHODS

The investigation was performed using the marine bacterial strain "*Alteromonas marinoglutinosa*" NCIMB 1770 from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland. The bacteria were isolated from superficial layers of coastal seawaters near the coast of California (USA) and were first identified as "*Pseudomonas marinoglutinosa*" [2]. The further studies using DNA–RNA hybridization showed that this species is related to the *Alteromonas* genus [3]. Bacteria were grown in the medium of Youschimizu and Kimura [4].

Lipopolysaccharide was isolated from dry bacterial cells by extraction with hot aqueous phenol [5]. Nucleic acids were removed by precipitation with trichloroacetic acid at pH 2. The precipitate was removed by centrifugation and the supernatant was dialyzed against distilled water and lyophilized. The resulting lipopolysaccharide was cleaved in 1% acetic acid for 3 h at 100°C, the precipitate of lipid A was removed by centrifugation, and the polysaccharide fraction was separated by gel chromatography on Sephadex G-50 (M) (Pharmacia, Sweden) 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.

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

Complete acid hydrolysis of the polysaccharide was performed 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). Downward 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 as described earlier [6]. For amino sugar identification, the polysaccharide was hydrolyzed with 4 M hydrochloric acid (for 4 h at 100°C), and then the hydrolyzate was analyzed using an LKB Biochrom 4151 ALPHA PLUS amino sugar analyzer (Sweden) fitted with a 200 × 4.6-mm column with Ultra Pak (8 μ) resin in 0.2 M sodium citrate (pH 6.46), at 50-85°C.

Absolute configurations of monosaccharides were determined by measuring their specific optical rotations on a Perkin-Elmer model 141 instrument. Free monosaccharides were isolated from the polysaccharide hydrolyzate using downward preparative paper chromatography.

Methylation was performed according to Hakomori [8]. Acetates of partially methylated methylglycosides were prepared by a standard procedure and analyzed by GLC [6] and GLC–mass spectrometry. GLC–mass spectrometry was performed on a Hewlett Packard 5890 instrument (USA) equipped with a glass capillary column coated with 5MS 5% Phenyl Methyl Siloxane stationary phase and connected to a Hewlett Packard 5973 mass spectrometer (USA) for the temperature range 120-225°C at 1°C/min.

^{13}C-NMR and ^{1}H-NMR spectra were measured with a Bruker AC-250 spectrometer (Germany) in D_2O solutions at 20°C using methanol (δ = 50.15 ppm) and dimethylsulfoxide (δ = 39.6 ppm) as the internal standards.

RESULTS AND DISCUSSION

Lipopolysaccharide was isolated from dry bacterial cells by extraction with hot aqueous phenol [5]. O-Specific polysaccharide was prepared by mild acid hydrolysis of the lipopolysaccharide followed by chromatography of the polysaccharide fraction on Sephadex G-50.

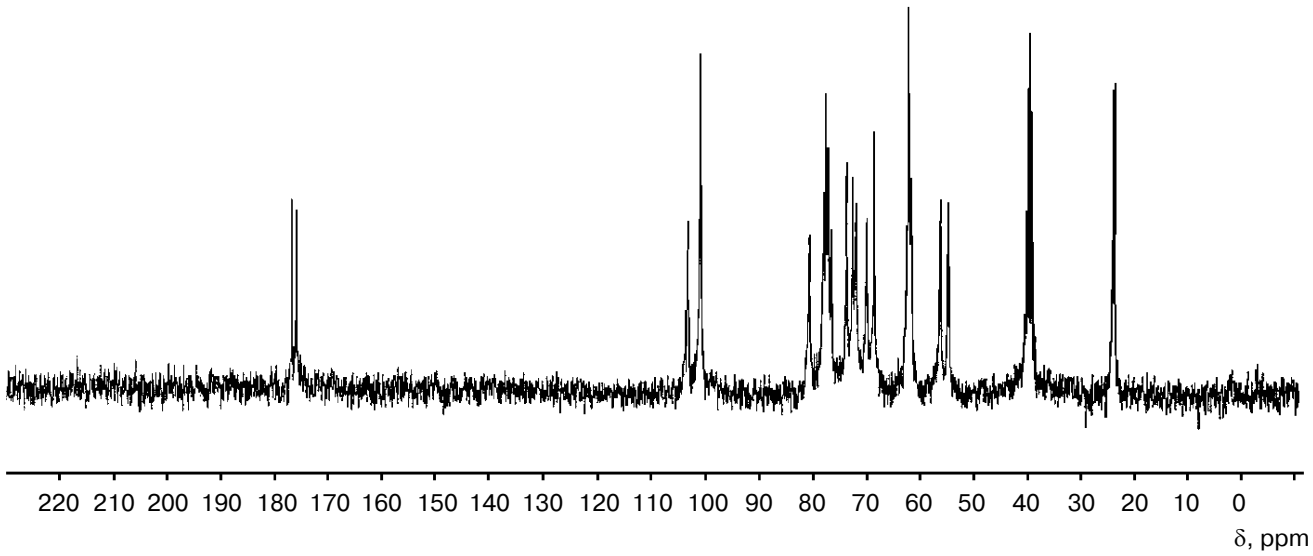
Paper chromatography and GLC of the hydrolyzate of the O-specific polysaccharide demonstrated that it contains galactose, mannosamine, and glucosamine in equimolar ratio. The presence of mannosamine and glucosamine was confirmed using an amino acid analyzer. All three monosaccharides were individually obtained by preparative paper chromatography. Their specific optical rotations were determined: D-galactose, $[\alpha]^{20}_{578}$ +76° (0.2 g per 100 ml water); N-acetyl-D-mannosamine, $[\alpha]^{20}_{578}$ −7° (0.25 g per 100 ml water), and N-acetyl-D-glucosamine $[\alpha]^{20}_{578}$ +67.2° (0.2 g per 100 ml water) (compare with data in [7]). Based on the specific optical rotations, the isolated monosaccharides were shown to have D-configuration.

The ^{13}C-NMR spectrum of the O-specific polysaccharide (D_2O, internal standard DMSO-d_6) indicated that its repeating unit was regular and had the size of a trisaccharide (figure). The spectrum had signals of three anomeric carbon atoms at 103.2 and 100.8 ppm (the latter with double integrated intensity), characteristic signals of N-acetyl groups at 24.0, 23.7 ppm (CH_3-) and 175.0, 175.9 ppm (CO-), two signals of C-atoms bearing nitrogen at 54.7 ppm ($J_{C2\text{-}H2}$ = 144 Hz) and 56.2 ppm ($J_{C2\text{-}H2}$ = 139 Hz), and two signals of hydroxymethyl groups (61.8 and 62.1 ppm). The total number of signals was 16, signals at 100.8 and 68.2 ppm having double integrated intensity.

Thus, the polysaccharide is constructed from trisaccharide repeating units including one residue each of D-galactose, N-acetyl-D-mannosamine, and N-acetyl-D-glucosamine.

The $^{1}J_{C,H}$ coupling constant values for anomeric atoms were determined from the gated-decoupling ^{13}C-NMR spectrum of the polysaccharide. Relatively small constants ($J_{C1\text{-}H}$ = 159 Hz and $J_{C1\text{-}H}$ ~160 Hz) for the signals at 103.2 and 100.8 ppm, respectively, showed that monosaccharides are β-linked, and relatively large constant ($J_{C1\text{-}H}$ ~170 Hz) for the signal 100.8 ppm suggested an α-linked monosaccharide [9]. The $^{1}J_{C,H}$ coupling constant values also showed that all sugar residues are in their pyranose form ($^{1}J_{C,H}$ coupling constant values of the furanose form is ~173-175 Hz [10]).

The character of substitution of monosaccharide residues in the O-specific polysaccharide was established using the methylation method [8]. Analysis of partially methylated methylglycoside acetates by GLC–mass spectrometry identified 2,6-di-O-methyl-D-galactopyranoside, permethylated 2-acetamido-2-deoxy-D-mannopyranoside, and 4,6-di-O-methyl-2-(N-methyl)-acetamido-2-deoxy-D-glucopyranoside. The data given indicated that the repeating unit of the O-specific polysaccharide is a branched trisaccharide. The main chain of the polysaccharide has the residue of D-galactose with



^{13}C-NMR spectrum of the O-specific polysaccharide of "*A. marinoglutinosa*" NCIMB 1770

substitutions in positions 3 and 4, and residue of 2-acetamido-2-deoxy-D-glucose replaced in position 3. The 2-acetamido-2-deoxy-D-mannose residue is located in a branch of the chain.

To obtain more information on the structure of the branched chain, the polysaccharide was subjected to Smith degradation followed by gel filtration on TSK-HW-40 gel. This resulted in isolation of a modified polysaccharide containing D-galactose and 2-acetamido-2-deoxy-D-glucose residues.

Methylation of modified polysaccharide and GLC–mass spectrometric analysis of methylation products as partially methylated methylglycoside acetates revealed 2,4,6-tri-O-methyl-D-galactose and 4,6-di-O-methyl-2-acetamido-2-deoxy-D-glucose. It follows that the residue of 2-acetamido-2-deoxy-D-mannose connects with the residue of D-galactose in position 4. The fact that mixtures resulting from the methanolysis of both the original and the modified polysaccharides contain small amounts 2,3,4,6-tetra-O-methyl-D-galactopyranoside indicated that the D-galactose residue is located in the non-reducing terminus of the polysaccharide chain.

In the anomeric region of the ^{13}C-NMR spectrum of the modified polysaccharide (figure and table), there are two signals of carbon atoms, at 103.7 and 100.8 ppm. In addition, in the spectrum there are characteristic signals of

Data of ^{13}C-NMR spectroscopy for the O-specific polysaccharide of "*A. marinoglutinosa*" NCIMB 1770

Residue	Chemical shift (δ), ppm					
	C1	C2	C3	C4	C5	C6
Polysaccharide						
β-D-Man*p*NAc 1 ↓ 4	100.8	54.7	73.7	68.5	77.3	61.6
→3)-α-D-Gal*p* (1→	100.8	71.9	78.1	77.6	72.5	62.1
→3)-β-GlcNAc*p* (1→	103.2	56.2	80.7	69.9	76.6	62.1
Modified polysaccharide						
→3)-α-D-Gal*p* (1→	100.2	71.7	80.2	68.6	71.7	61.6
→3)-β-GlcNAc*p* (1→	103.7	55.5	80.4	70.0	76.6	61.6

the N-acetyl group at 23.6 (CH_3-) and 174.0 (C=O) ppm and the signal of a carbon atom bearing nitrogen at 55.5 ppm. These data are consistent with the chemical data and confirm that the modified polysaccharide contains D-galactose and 2-acetamido-2-deoxy-D-glucose residues. It should be noted that the chemical shift of the C2-atom of the 2-acetamido-2-deoxy-D-glucose residue (55.5 ppm) is characteristic for the β-2-acetamido-2-deoxy-D-glucose residue glycosylated of the α-glycoside bond at C3-atom [11]. Based on these results and published data, the signals of C-atoms of the [-3-β-D-Glc*p*NAc-] residue are unequivocally allocated, other signals of the spectrum (at δ: 100.2, 80.4, 71.7, 71.6, 68.6, and 61.6) corresponding to an α-D-Gal residue. For more precise reference of signals of the galactose residue, ^{1}H-NMR spectrometry of modified polysaccharide was performed. In the ^{1}H-NMR spectrum of modified polysaccharide, protons H1 and H4 of the galactose residue resonate at δ = 5.44 ppm (J = 3.5 Hz) and δ = 4.19 ppm (J = 6 Hz), respectively. On selective irradiation of proton H4 (δ = 4.19 ppm), it was determined to correspond to the signal at δ = 68.4 ppm in the ^{13}C-NMR spectrum. This indicates glycosylation of the residue α-D-Gal*p* at the C3 atom. Additional confirmation of glycosylation of the D-Gal residue at the C3 atom was obtained from the chemical shift of the signal of the C1 atom of the -β-D-Glc*p*NAc residue. The C1 atom of the residue -β-D-Glc*p*NAc for the fragment -β-D-Glc*p*NAc1→3-α-D-Gal*p* is known to resonate at δ = 102.4 ppm, and in case of the fragment -β-D-Glc*p*NAc1→4-α-D-Gal*p* at δ = 101.1 ppm [12]. Thus, the data indicate that the modified polysaccharide has the following structure of the repeating unit:

[→3)-α-D-Gal*p*(1→3)-β-D-Glc*p*NAc(1→].

Based on the chemical analysis data, the O-specific polysaccharide is branched and there is a residue of 2-acetamido-2-deoxy-D-mannose in a side chain. The results of the ^{13}C-NMR spectral analysis indicate its β-configuration. Based on published data [13], our results allow the allocation of signals of C atoms of the β-D-Man*p*NAc residue (table) in the spectrum of the native polysaccharide. Comparison of the ^{13}C-NMR spectra of the native and modified polysaccharides shows that the chemical shifts of C atoms of the β-D-Glc*p*NAc residue have no significant deviations; hence, a β-D-Man*p*NAc residue glycosylates the α-D-Gal*p* residue. From the spectrum of the native polysaccharide it is also seen that the signal of the C4 atom of the α-D-Gal*p* residue is shifted to comparatively weak magnetic field by Δδ = 9.2 ppm, and the signal of the C3 atom to comparatively strong magnetic field by Δδ = 2.2 ppm with respect to their position in the spectrum of the modified polysaccharide. This suggests that in the native polysaccharide the α-D-Gal*p* residue has substituents at the C3 and C4 atoms, which is in consistent with the chemical data.

Thus, based on all our data, we suggest the following structure of the repeating unit of the O-specific polysaccharide:

β-D-Man*p*NAc
1
↓
4
→3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→

This structure as well as the structure of the O-specific polysaccharide from *P. marinoglutinosa* KMM 232 [1] appreciably differs from structures of other representatives of *Alteromonas* and *Pseudoalteromonas* genera. Both bacterial strains are isolated from seawater, but from different depths. Their polysaccharides are constructed from small branched repeating units that represent disaccharide, and side branch of one of them (KMM 232) has a sulfate group, the other has mannosamine. A common feature of the major polysaccharides of other representatives of these two genera is their acidic character and also the presence of rare sugars and non-carbohydrate residues ([14] and references therein).

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