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STRUCTURE OF CARBOHYDRATE ANTIGENS FROM *Microbulbifer* sp. KMM 6242

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A capsular polysaccharide (CPS) containing D-galactosamine uronic acid and D-alanine was isolated from a culture of the marine proteobacterium Microbulbifer *sp. KMM 6242. 2D NMR spectroscopy showed that the CPS is a homopolymer of 2-acetamido-2-deoxy-*N*-(D-galacturonyl)-D-alanine with the structure →4)-β-D-GalpNAcA6(D-Ala)-(1→. An* O*-specific polysaccharide containing D-ribose and D-galactose was isolated from the cell-membrane lipopolysaccharide. 1D and 2D NMR spectroscopy established the structure of the disaccharide repeating unit of the polysaccharide as →3)-β-D-Ribf-(1→4)-β-D-Galp-(1→.*

Keywords: *Microbulbifer*, capsular polysaccharide, *O*-specific polysaccharide, NMR spectroscopy.

Marine proteobacteria are typical procaryotes that occupy various ecological niches and represent a significant part of the Pacific microbial community. Bacteria of the genus *Microbulbifer* are typical examples of marine aerobic Gram-negative heterotrophic microorganisms that were first described as an independent taxon in 1997 and were assigned to the γ-subclass of the class Proteobacteria [1]. They are capable of forming capsules in liquid medium and produce a broad spectrum of hydrolytic enzymes. At present the genus numbers 14 valid described species, strains of which were isolated from various marine sources [2]. The composition and structure of carbohydrate-containing biopolymers from the cell membrane of representatives of this genus have not been studied. Herein we present results from a structural study of carbohydrate antigens from marine proteobacteria [3, 4] and establish the structure of a capsular polysaccharide (CPS) and *O*-specific polysaccharide (OPS) from the marine microorganism *Microbulbifer* sp. strain KMM 6242.

The CPS was isolated from bacterial biomass raw material by extraction with NaCl (0.9%, 3×) and purified of ballast protein by treatment with aqueous phenol (45%). Lipopolysaccharide (LPS) was isolated by extraction with aqueous phenol using the Westphal method [5], purified of nucleic-acid impurities by precipitation with trichloroacetic acid at pH 2, and fractionated into carbohydrate and lipid constituents by mild acid degradation. Gel chromatography of the polysaccharide fraction over TSK-50(F) sorbent produced the OPS.

Carbohydrate analysis of the CPS and OPS as the polyol acetates and methylglycoside acetates using PC and TLC showed the presence in the CPS of galactosamine uronic acid and alanine; in the OPS, ribose and galactose. All constituents were isolated pure and assigned as the D-isomers based on their specific optical rotations. The nature of the monosaccharide substitution in the OPS was determined by methylation [6]. GC–MS of the acetates of partially methylated polyols identified 3-*O*-acetyl-2,4,5-tri-*O*-methylpentose and 4-*O*-acetyl-2,3,6-tri-*O*-methylhexose, which indicated the presence of 1,3-bound D-ribose units and 1,4-bound D-galactose units in the polysaccharide chain.

The ^{13}C NMR spectrum (Table 1) of the CPS showed resonances for a single anomeric C atom at 101.1 ppm; two C atoms bonded to N at 52.2 and 55.2; two deoxy groups at 17.6 and 22.9 (CH_3-acetamide), C=O groups at 169.2, 175.2, and 179.7; and three resonances of ring C atoms at 72–78. Analogously the PMR spectrum of the CPS contained resonances for an anomeric proton at 4.61 ppm ($J_{1,2}$ = 7 Hz); two protons on C atoms bonded to N at 3.90 and 4.40; and two resonances for CH_3 groups at 2.10 (NAc) and 1.45 (J ~ 7 Hz). The SSCC of the anomeric C atom that was determined from the ^{13}C NMR spectrum taken without C–H decoupling (gated decoupling experiment) was 165 Hz for the resonance at 101.1 ppm. This was consistent with the β-configuration for this moiety [7] and confirmed the SSCC of the anomeric proton $J_{H1,H2}$ = 7 Hz [8].

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TABLE 1. NMR Spectra of CPS and OPS from *Microbulbifer* sp. KMM 6242 (δ, ppm)

Unit	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
			CPS			
→4)-*β*-D-Gal*p*NAcA-(1→*	101.1	55.2	72.0	77.2	74.6	169.1
	4.61	3.90	3.80	4.06	4.04	
D-Ala	179.7	52.2	17.6			
		4.40	1.45			
β-D-GalNAcA**	95.81	54.46	72.02	70.34	76.49	175.64
	4.71	3.96	3.87	4.32	4.10	
→4)-*β*-D-GalNAcA-(1→	101.99	53.14	70.33	79.34	75.25	169.1
	4.58	4.09	4.01	4.56	4.26	
			OPS			
→3)-*β*-D-Rib*f*-(1→	108.5	74.9	80.5	81.7	63.9	
	5.29	4.31	4.35	4.18	3.85/3.83	
→4)-*β*-D-Gal*p*-(1→	104.0	71.2	76.9	77.3	75.1	61.4
	4.55	3.66	3.88	4.74	3.89	3.78

*Additional resonances of *N*-acetyl group: δ 22.9 (CH_3) and δ 175.2 (C=O); **literature data [11].

Spectra of the CPS were interpreted using homonuclear COSY and TOCSY and heteronuclear ^1H–^{13}C HSQC experiments.

The spin system of the monosaccharide unit was identified for ring protons in COSY and TOCSY spectra. The correlation H1–H2–H3, which is typical for the *galacto*-configuration of the monosaccharide unit, was observed in the TOCSY spectrum. The correlation H2/C2 at 3.90/55.2 ppm was found in the HSQC spectrum and is characteristic of 2-amino-2-deoxysugars. The lack of a resonance for a hydroxymethyl group and the presence of one for a COOH group in the ^{13}C NMR spectrum of the CPS confirmed that the monosaccharide unit was *β*-galactosamine uronic acid. The strong-field position of the carbonyl resonance at 169.1 ppm relative to its usual position at 175–176 was typical of an amide bond [9, 10]. Thus, the alanine unit was located in the 6-position of the amine uronic acid. Furthermore, the monosaccharide C1, C5, and C6 resonances were not shifted if the ^{13}C NMR spectrum was recorded at pD 2. This also confirmed that the carboxyl was substituted [9].

The C4 resonance in the ^{13}C NMR spectrum of the CPS was shifted by +9 ppm compared with its position in the spectrum of the unsubstituted monosaccharide (glycosylation *α*-effect) [8, 11]. This indicated that the sugar unit was substituted in the 4-position so that the CPS had the following structure:

→4)-*β*-D-Gal*p*NA6(D-Ala)-(1→

Table 1 gives the complete assignment of resonances in the spectra.

The ^{13}C NMR spectrum of the OPS (Table 1) showed resonances of two anomeric C atoms at 108.5 and 104.0 ppm; of two hydroxymethyls at 63.9 and 61.4; and of seven ring C atoms at 70–82. The SSCC of the anomeric C atom was determined from the ^{13}C NMR spectrum taken without C–H decoupling (gated decoupling experiment) and was 165 Hz for the resonance at 104.5 ppm. This was consistent with the *β*-configuration of the corresponding unit [7]. The chemical shift of 109.0 ppm and its SSCC (179 Hz) were characteristic of a *β*-furanoside monosaccharide unit [12]. A test for bonded protons (APT-experiment [13]) showed a lack of 1,6-bonded monosaccharide units.

The PMR spectrum of the OPS contained resonances for two anomeric protons at 5.29 ppm (s, $J < 2$ Hz) and 4.55 (d, $J \sim 8$ Hz). This also confirmed the anomeric configuration of the sugars and the sizes of their rings [8].

The NMR spectra of the OPS were completely interpreted using 2D spectroscopy (Table 1) including homonuclear ^1H–^1H-COSY, TOCSY, and ROESY and heteronuclear ^1H–^{13}C HSQC according to the usual methodology [14]. Resonances of *β*-Rib C-3 and *β*-Gal C-4 in the ^{13}C NMR spectrum of the OPS were shifted to weak field at 80.5 and 77.3 ppm compared with their positions in spectra of the unsubtituted monosaccharides [8]. This indicated that the correspondig hydroxyls were glycosylated and confirmed the substitution pattern of the monosaccharide units that was established by methylation. The ^1H–^{13}C HSQC spectrum of the OPS showed the following correlation resonances for the D-ribose: H1(δ 5.29)Rib/C1(δ 108.5)Rib; H2(δ 4.31)Rib/C2(δ 74.9)Rib; H3(δ 80.5)Rib/C3(δ 80.5)Rib; H4(δ 4.18)Rib/C4(δ 81.7)Rib; H5(δ 3.85)Rib/C5(δ 63.9)Rib;

for D-galactose: H1(δ 4.55)Gal/C1(δ 104.0)Gal; H2(δ 3.66)Gal/C2(δ 71.2)Gal; H3(δ 3.88)Gal/C3(δ 76.9)Gal; H4(δ 4.74)Gal/C4(δ 77.3)Gal; H5(δ 3.84)Gal/C5(δ 75.2)Cal. It should be noted that the Gal C3 resonance appeared at unusually weak field at 76.9 ppm (glycosylation *β*-effect +2.8 ppm). This is probably explained by conformational features of the glycosylated *β*-D-ribofuranose.

The sequence of monosaccharide units in the repeating unit was established using correlations between the anomeric protons and those of the glycosylated C atoms in the ROESY spectrum: H1(δ 6.29)Rib/H4(δ 4.74)Gal and H1(δ 4.55)Gal/H3(δ 4.35)Rib. Thus, the structure →3)-*β*-D-Rib*f*-(1→4)-*β*-D-Gal*p*-(1→ was proposed for the repeating unit of the OPS based on the results.

EXPERIMENTAL

The strain *Microbulbifer* sp. KMM 6242 from the Marine Microorganism Collection of the PIBOC, FEB, RAS, was isolated from the sea urchin *Strongylocentrotus intermedius* (Troits Bay, Peter the Great Gulf, Sea of Japan) and isolated as a separate colony on agar medium after incubation for seven days at 28°C. Microorganisms were cultured on a shaker (120 rpm) for 60 h at room temperature in liquid growth medium containing (g/L) peptone (5.0), yeast extract (2.0), glucose (1.0), K_2HPO_4 (0.2), $MgSO_4$ (0.05), distilled water (500 mL), and marine water (500 mL) at pH 7.8. Bacteria biomass was separated by centrifugation at 5,000 rpm. ^{13}C NMR spectra were recorded in D_2O at 60°C on a Bruker Avance DPX-300 instrument with MeOH as an internal standard (δ_C 50.15 ppm). 2D NMR spectra were taken in D_2O on a Bruker Avance DRX-500 spectrometer with acetone (δ_H 2.225 ppm) internal standard. Solutions were lyophilized or evaporated in vacuo. Optical rotation was measured on a Perkin–Elmer 141 instrument. Descending chromatography was carried out on Filtrak FN-15 and Whatman 3MM paper using *n*-BuOH:Py:H_2O (6:4:3) with detection of monosaccharides by basic $AgNO_3$ and ninhydrin (5%) in acetone. Gel chromatography was performed over a column (2.5 × 100 cm) of TSK HW 50 (F) gel using acetic acid (0.3%). Elution curves were plotted using a RIDK 101 differential refractometer (Czech Rep.). GC was carried out on a Hewlett–Packard 6850 chromatograph with a capillary column (30 m × 0.4 mm) and temperature gradient 150→230°C at 3°/min. GC–MS was carried out under the same conditions.

Isolation of Polysaccharides. Moist biomass (~20 g) was extracted with NaCl solution (0.9%, 3×) using ultrasound (44 kHz, 15 min). Cells were separated by centrifugation. The extracts were combined, dialyzed, concentrated, and treated with phenol (45%, 65°C, 30 min). The reaction mixture was dialyzed, concentrated, and lyophilized. Yield of CPS, 120 mg. The remaining cells were treated by the Westphal method (3×) [5]. The aqueous phase was dialyzed, concentrated, and lyophilized. Yield of LPS, 550 mg. The LPS (500 mg) was hydrolyzed by AcOH (1%, 100°C, 3 h). The precipitate of lipid A was separated by centrifugation (90 mg). The polysaccharide fraction was separated by gel chromatography over TSK HW 50 (F) gel to afford the OPS (220 mg).

Isolation of Monosaccharides. CPS (20 mg) was hydrolyzed by HCl (4M, 100°C, 4 h). The hydrolysate was separated by preparative PC to afford D-galactosamine uronic acid (2 mg), $[\alpha]_D$ –25° (*c* 0.2, 2.5% HCl) (lit. [15] $[\alpha]_D$ –31.9°) and D-alanine (3 mg), $[\alpha]_D$ –10° (*c* 0.3, 2.5% HCl) (lit. [16] $[\alpha]_D$ –14.2°, *c* 6.0, 1N HCl).

OPS (20 mg) was hydrolyzed by CF_3COOH (2M, 100°C, 3 h). The hydrolysate was separated by preparative PC to afford D-ribose (4 mg), $[\alpha]_D$ –20° (*c* 0.4, H_2O) (lit. [15] $[\alpha]_D$ –23.7°) and D-galactose (6 mg), $[\alpha]_D$ +76° (*c* 0.6, H_2O) (lit. [15] $[\alpha]_D$ +80.5°).

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