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Note

Structure of the O-polysaccharide chain of the lipopolysaccharide of *Psychrobacter muricolla* 2pS^{T} isolated from overcooled water brines within permafrost

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ABSTRACT

Psychrotrophic bacteria of the genus *Psychrobacter* have not been studied in respect to lipopolysaccharide structure. In this work, we determined the structure of the O-specific polysaccharide of the lipopolysaccharide of *Psychrobacter muricolla* 2pS^{T} isolated from overcooled (−9 °C) water brines within permafrost. The polysaccharide was found to be acidic due to the presence of an amide of 2-acetamido-2-deoxy-L-guluronic acid with glycine (L-GulNAcA6Gly), which has not been hitherto found in nature. The following structure of the disaccharide repeating unit of the polysaccharide was established using composition analysis along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy:

→4)-α-L-Gul*p*NAcA6Gly-(1→3)-β-D-Glc*p*NAc-(1→

The genus *Psychrobacter* from the family Moraxellaceae within the γ-subclass of Proteobacteria comprises psychrophilic to psychrotolerant, halotolerant aerobic non-motile Gram-negative coccobacilli.[1] *Psychrobacter* species are isolated from a variety of low-temperature environments including Antarctic sea ice, soil and sediments as well as the deep sea.[2–4]

Lipopolysaccharide is the major component of the outer membrane of Gram-negative bacteria and has been known as a pathogen-associated molecular pattern recognized by receptors of the immune system. It consists of three structural domains: an O-specific polysaccharide consisting of oligosaccharide repeats (O-units), a central oligosaccharide (core) and lipid A. The O-specific polysaccharide chain of the lipopolysaccharide (O-polysaccharide, O-antigen) protruding into the surroundings of the bacterial cell is the most variable cell surface constituent, and its structural diversity is believed to be important for adaptation of bacteria for specific niche. Until now, lipopolysaccharide structure has not been studied in the genus *Psychrobacter*.

In this work, we report on the structure of the O-polysaccharide chain of the lipopolysaccharide of *Psychrobacter muricolla* 2pS^{T}.[5] The strain has been isolated in Kolyma lowland (Siberia) from the lens of overcooled (−9 °C) highly saline (13%) water brine intersected by borehole 16/99 at a depth of 11 m within a permanently frozen marine layer that was deposited beneath shallow lagoons at temperatures slightly above 0 °C and froze subaerially as the Polar Ocean regressed 110,000–112,000 years ago.[6–8]

The lipopolysaccharide was obtained from dried bacterial cells by the phenol–water procedure[9] and degraded under mild acidic conditions. The resultant high-molecular mass O-polysaccharide was isolated by GPC on Sephadex G-50. Full acid hydrolysis of the polysaccharide followed by analysis using an amino acid analyzer revealed glucosamine and glycine. The former was confirmed by GLC of the alditol acetates, and analysis by GLC of the (*S*)-2-octyl glycosides showed that GlcN has the D-configuration. The second sugar component of the O-polysaccharide was not seen in sugar analysis and was identified by NMR spectroscopy as 2-amino-2-deoxy-L-guluronic acid (L-GulNA).

The ^{1}H NMR spectrum of the O-polysaccharide showed three signals in the region of anomeric protons, other sugar protons in

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the region δ 3.41–4.29 and two N-acetyl groups at δ 1.97 and 2.06. However, only two signals for anomeric carbons were observed in the ^{13}C NMR spectrum. An ^{1}H,^{13}C HSQC experiment (Fig. 1) revealed two H-1,C-1 cross-peaks at δ 5.11/99.2 and 4.51/101.9, whereas the third low-field signal in the ^{1}H NMR spectrum at δ 4.96 gave a correlation with a non-anomeric carbon at δ 67.1 and was assigned to H-5 of GulNA (see below). The ^{13}C NMR spectrum also showed signals for one OCH_2–C group at δ 61.9 (C-6 of GlcNAc), one NCH_2–C group at δ 43.2 (C-2 of glycine), two more nitrogen-bearing carbons at δ 46.9 and 56.5 (C-2 of GlcN and GulNA), other sugar ring carbons at δ 68.5–80.5, CH_3 of two N-acetyl groups at δ 23.4 and 23.6, and CO groups of NAc, glycine (C-1) and GulNA (C-6) at δ 172.6–175.5.

Therefore, the O-polysaccharide has a disaccharide repeating unit containing one residue each of GlcN, GulNA, Gly and two NAc groups.

The ^{1}H and ^{13}C NMR spectra of the O-polysaccharide were assigned using 2D ^{1}H,^{1}H COSY, ROESY, ^{1}H,^{13}C HSQC and HMBC experiments (Table 1), and spin systems for two sugar residues and glycine were recognized. Correlations of H-2 to nitrogen-bearing carbons (C-2) at δ 3.82/56.5 and 4.29/46.9, respectively, in the ^{1}H,^{13}C HSQC spectrum showed that both constituent monosaccharides are 2-amino sugars. That one from them is an amino uronic acid was confirmed by a correlation between H-5 and the carboxyl group (C-6) at δ 4.96/172.6 revealed by an ^{1}H,^{13}C HMBC experiment. The C-2 chemical shift (δ 46.9) indicated its *gulo* configuration (compare published data δ 46.9–47.0 for C-2 of GulNAcA,[10] whereas all other stereoisomers are characterized by a significantly lower-field position of the C-2 signal[11]).

Glycine was demonstrated by correlations of H-2a,2b at δ 3.92 and 4.01 to a nitrogen-bearing carbon (C-2) at δ 43.2 in the ^{1}H,^{13}C HSQC spectrum and to a CO group (C-1) at δ 175.5 in the HMBC spectrum. A correlation between H-2a,2b of Gly and C-6 of GulNA at δ 3.92, 4.01/172.6 in the HMBC spectrum indicated that Gly is amide-linked to the carboxyl group of GulNA. Therefore, the amino groups of GlcN and GulNA are acetylated.

The β-configuration of GlcNAc was inferred from the H-1 chemical shift of δ 4.51 and strong H-1,H-3 and H-1,H-5 correlations in the ROESY spectrum. A H-1,H-2 correlation with no H-1,H-3 and H-1,H-5 correlations in this spectrum showed that GulNAcA is α-linked.

Downfield displacements of the signals for C-3 of GlcNAc and C-4 of GulNAcA to δ 80.5 and 77.6, as compared with their positions in β-Glc*p*NAc and α-Gul*p*NAc at δ 75.1[12] and 69.6,[13] respectively, revealed the glycosylation pattern of the monosaccharides. It was confirmed by interresidue GlcNAc H-1,GulNAcA H-4 and GulNAcA H-1,GlcNAc H-3 cross-peaks at δ 4.51/4.21 and δ 5.11/3.71, respectively, in the ROESY spectrum. These data also showed that the O-polysaccharide is linear.

Glycosylation with β-D-Glc*p*NAc at position 4 caused a relatively small upfield effect of −1.9 ppm on C-3 of α-Gul*p*NAcA (a shift from δ 70.4 in non-substituted α-Gul*p*NAc[13] to δ 68.5 in the O-polysaccharide). Such a small effect is typical of different absolute configurations of the linked monosaccharides, that is the L-configuration of GulNAcA (compare a much higher negative glycosylation effect of −3.9 ppm in case of the same absolute configuration of the constituent monosaccharides[14]). The absolute configuration of GulNAc was confirmed by a similarity of the ^{13}C NMR chemical shift of this monosaccharide in the β-D-Glc*p*NAc-(1→4)-α-Gul*p*NAcA disaccharide fragment of the polysaccharide studied and the β-D-Man*p*NAcA-(1→4)-α-L-Gul*p*NAcA fragment of the capsular polysaccharide of *Neisseria meningitides* group I.[13]

Therefore, the O-polysaccharide of *P. muricolla* 2pS^{T} has the structure shown in Chart 1. Its peculiar feature is the presence of an amide of L-GulNAcA with glycine. To the best of our knowledge, only the primary amide of α-L-GulpNAcA has been hitherto reported in nature, as a component of the O-polysaccharide of *Pseudomonas tolaasii* NCPPB 2192.[15] Non-amidated 4-substituted α-L-GulpNAcA has been found in a number of bacterial polysaccharides, including O-polysaccharides of *Pseudomonas* sp. OX1,[16] *Halomonas magadii* (now *magadiensis*) 21 MI,[17] *Acinetobacter haemolyticus* 57 and 61,[10] *Pseudoalteromonas nigrifaciens* KMM 158 and KMM 161,[18] and the capsular polysaccharide of *Neisseria meningitides* group I[13]; the O-polysaccharide of *Idiomarina zobellii* KMM 231^{T} contains α-L-Gul*p*NA with the free amino group.[19]

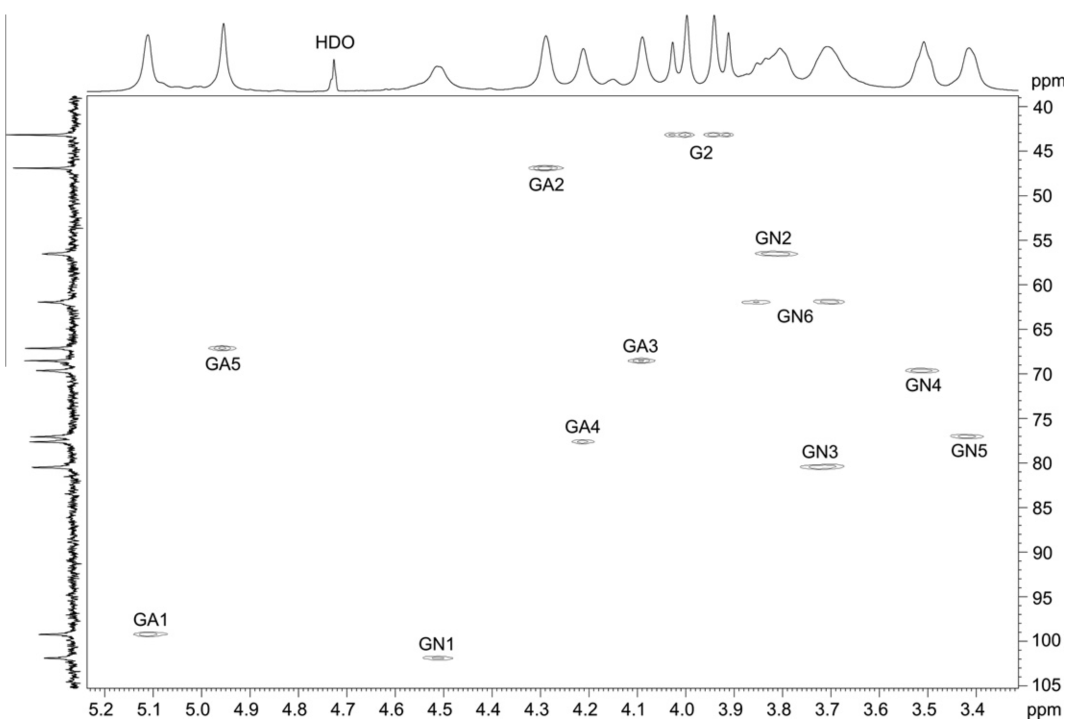

Figure 1. Part of a ^{1}H,^{13}C HSQC spectrum of the O-polysaccharide of *P. muricolla* 2pS^{T}. *Abbreviations*: N, GlcNAc; GA, GulNAcA; G, glycine.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the O-polysaccharide of *P. muricolla* $2pS^T$

Sugar residue	Nucleus	1	2 (2a, 2b)	3	4	5	6 (6a, 6b)
→3)-β-D-Glc*p*NAc-(1→	^{1}H	4.51	3.82	3.71	3.51	3.41	3.07, 3.85
	^{13}C	101.9	56.5	80.5	69.6	77.0	61.9
→4)-α-L-Gul*p*NAcA-(1→	^{1}H	5.11	4.29	4.15	4.21	4.96	
	^{13}C	99.2	46.9	68.5	77.6	67.1	172.6
Gly	^{1}H	175.5	3.92, 4.01				
	^{13}C		43.2				

Chemical shifts for NAc are: δ_H 1.97 and 2.06; δ_C 23.4, 23.6 (both CH_3) and 175.0 (2CO).

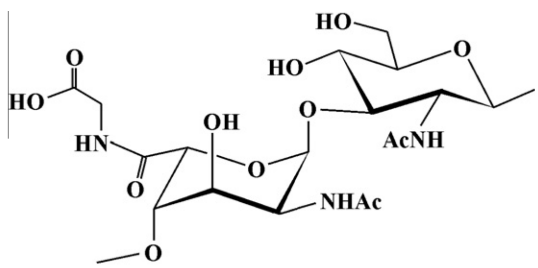

Chart 1. Structure of the O-polysaccharide of *P. muricolla* $2pS^T$.

1. Experimental

1.1. Bacterial strain and cultivation of bacteria

Psychrobacter muriicola strain $2pS^T$ was provided by the All-Russian Collection of Microorganisms (B-2269 Type strain). Bacteria were grown to late log phase at 24 °C in a medium pH 7.2 containing (per 1 L distilled water): 4 g yeast extract; 1.12 g Na_2HPO_4; 0.4 g KH_2PO_4; 5 g NaCl; 2 g NH_4Cl; 1 g $MgSO_4{\cdot}7H_2O$; 0.01 g $CaCl_2$; 0.005 g $FeSO_4{\cdot}7H_2O$; 10 mL micronutrient solution SL-10.[20] Bacterial cells were washed and dried as described.[21]

1.2. Isolation of the lipopolysaccharide and O-polysaccharide

Lipopolysaccharide was isolated from dried bacterial mass (4 g) by the phenol–water procedure[9] followed by dialysis of the extract without layer separation. After removal of insoluble contaminations by centrifugation, the solution was freed from proteins and nucleic acids by treatment with cold (4 °C) aq 50% CCl_3CO_2H, the precipitate was removed by centrifugation, and the supernatant was dialyzed against distilled water and freeze-dried to yield lipopolysaccharide (352 mg).

A sample of the lipopolysaccharide (90 mg) was heated with 2% HOAc for 25 min at 100 °C, and a lipid precipitate was removed by centrifugation. The carbohydrate-containing supernatant was fractionated by GPC on a column (60 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in pyridinium acetate buffer (4 mL pyridine and 10 mL concd HOAc in 1 L water) monitored using a differential refractometer (Knauer, Germany) to give a high-molecular-mass O-polysaccharide (41 mg).

1.3. Composition analyses

For monosaccharide analysis, a sample of the O-polysaccharides (1 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), dried under a stream of nitrogen and reduced with an excess of $NaBH_4$ in water. After adding concd HOAc, evaporation and co-evaporation with 10% HOAc in MeOH (2 × 1 mL), the sample was acetylated with Ac_2O (0.5 mL, 100 °C, 20 min) and analyzed by GLC on an Agilent Technologies 7820A instrument with a HP-5 ms capillary column using a temperature gradient of 160 °C (3 min) to 290 °C at 7 °C min^{-1}. The absolute configuration of GlcN was determined by GLC of the acetylated (*S*)-2-octyl glycosides[22] under the same chromatographic conditions.

For analysis of amino components, a O-polysaccharide sample (2 mg) was hydrolyzed with 3 M CF_3CO_2H (120 °C, 3 h), the solution was evaporated, and the hydrolysate was analyzed using a Biotronik LC-2000 amino acid analyzer on a column (22 × 0.4 cm) of an Ostion LC AN B cation-exchange resin in 0.2 M sodium citrate buffer pH 3 at 65 °C.

1.4. NMR spectroscopy

An O-polysaccharide sample was deuterium-exchanged by freeze-drying twice from 99.9% D_2O and then examined as a solution in 99.95% D_2O at 30 °C on an Avance II 600 spectrometer (Bruker, Germany) using internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0.00) and acetone (δ_C 31.45) as references. 2D NMR spectra were obtained using standard Bruker software, and Bruker TopSpin 2.1 program was used to acquire and process the NMR data. Mixing times of 200 and 100 ms were used in TOCSY and ROESY experiments, respectively. Other NMR parameters were set essentially as described.[23]

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