Note

Structure of the O-polysaccharide chain of the lipopolysaccharide of Psychrobacter muricolla 2pST isolated from overcooled water brines within permafrost

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A R T I C L E  I N F O

Article history:
Received 19 October 2011
Received in revised form 24 November 2011
Accepted 27 November 2011
Available online 6 December 2011

Keywords:
Psychrobacter muricolla
Lipopolysaccharide
O-polysaccharide
Bacterial polysaccharide structure
2-Acetamido-2-deoxy-L-guluronic acid
Glycine

A B S T R A C T

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 2pST 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 1H and 13C NMR spectroscopy:

\[-4\)-α-L-GulNAcA6Gly-(1→3)-β-D-GlcNAc-(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. Psychrobacter species are isolated from a variety of low-temperature environments including Antarctic sea ice, soil and sediments as well as the deep sea. The 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 2pST. 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 laggoons at temperatures slightly above 0 °C and froze subaerially as the Polar Ocean regressed 110,000–112,000 years ago. The lipopolysaccharide was obtained from dried bacterial cells by the phenol–water procedure 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 α-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 1H 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 anomic carbons were observed in the 13C NMR spectrum. An 1H,13C 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 1H NMR spectrum at δ 4.96 gave a correlation with a non-anomic carbon at δ 67.1 and was assigned to H-5 of GulNA (see below). The 13C NMR spectrum also showed signals for one OCH2–C group at δ 61.9 (C-6 of GlcNAc), one CH2–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, CH3 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 1H and 13C NMR spectra of the O-polysaccharide were assigned using 2D 1H,1H COSY, ROESY, 1H,13C 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 1H,13C 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 1H,13C HMBC experiment. The C-2 chemical shift (δ 46.9) indicated its glyco configuration (compare published data δ 46.9–47.0 for C-2 of GalNAcA,10 whereas all other stereoisomers are characterized by a significantly lower-field position of the C-2 signal11).

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 1H,13C 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.

Therefore, the O-polysaccharide of P. muricolla 2pST 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, 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 GalNAc to δ 80.5 and 77.6, as compared with their positions in β-GalpNAc and α-GulpNAc at δ 75.112 and 69.613 respectively, revealed the glycosylation pattern of the monosaccharides. It was confirmed by interresidue GlcNAc H-1,GulNAc H-4 and GalNAc H-1,GulNAc 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-GlcpNAc at position 4 caused a relatively small upfield effect of −1.9 ppm on C-3 of α-GulpNAcA (a shift from δ 70.4 in non-substituted α-GulpNAc13 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 i-configuration of GalNAcA (compare a much higher negative glycosylation effect of −3.9 ppm in case of the same absolute configuration of the constituent monosaccharides14). The absolute configuration of GalNAcA was confirmed by a similarity of the 13C NMR chemical shift of this monosaccharide in the β-α-GlcNAc(1→4)-α-GulpNAcA disaccharide fragment of the polysaccharide studied and the β-α-ManpNAcA-(1→4)-α-L-GulpNAcA fragment of the capsular polysaccharide of Neisseria meningitides group I.13 Therefore, the O-polysaccharide of P. muricolla 2pST has the structure shown in Chart 1. Its peculiar feature is the presence of an amide of α-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 toladiensis 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 magadiensis (now magadiensis) 21 ML,17 Acinetobacter haemolyticus 57 and 61,10 Pseudoalteromonas nigricans KMM 158 and KMM 161,18 and the capsular polysaccharide of Neisseria meningitides group I13; the O-polysaccharide of Idiomarina zobellii KMM 2317 contains α-l-GulpNA with the free amino group.19
1. Experimental

1.1. Bacterial strain and cultivation of bacteria

_Psychrobacter muricola_ strain 2ps 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 NaHPO₄; 0.4 g K₂HPO₄; 5 g NaCl; 2 g NH₄Cl; 1 g MgSO₄·7H₂O; 0.01 g CaCl₂; 0.005 g FeSO₄·7H₂O; 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 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) aqueous 50% CCl₄·CO₂H, 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 x 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in pyridinium acetate buffer (4 mL pyridine and 10 mL conc 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 CF₃CO₂H (120°C, 2 h), dried under a stream of nitrogen and reduced with an excess of NaBH₄ in water. After adding conc HOAc, evaporation and co-evaporation with 10% HOAc in MeOH (2 x 1 mL), the sample was acetylated with Ac₂O (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 under the same chromatographic conditions.

For analysis of amino components, the O-polysaccharide sample (2 mg) was hydrolyzed with 3 M CF₃CO₂H (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 x 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% D₂O and then examined as a solution in 99.95% D₂O at 30°C on an Avance II 600 spectrometer (Bruker, Germany) using internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (δH 0.00) and acetone (δC 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

**Acknowledgments**

Authors thank Dr A. P. Arbatsky (Zelinsky Institute) for help with analysis on an amino acid analyzer. This work was supported by the Russian Foundation for Basic Research (Projects Nos. 10-04-00590 and 10-04-01470) and the fundamental research program Molecular and Cellular Biology of the Presidium of the Russian Academy of Sciences.

**References**


**Table 1**

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>Nucleus</th>
<th>1</th>
<th>2 (2a, 2b)</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6 (6a, 6b)</th>
</tr>
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<tbody>
<tr>
<td>(–)β-D-GlcNAc-1→</td>
<td>²H</td>
<td>4.51</td>
<td>3.82</td>
<td>3.71</td>
<td>3.51</td>
<td>3.41</td>
<td>3.07, 3.85</td>
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<tr>
<td></td>
<td>¹³C</td>
<td>101.9</td>
<td>56.5</td>
<td>80.5</td>
<td>69.6</td>
<td>77.0</td>
<td>61.9</td>
</tr>
<tr>
<td>(–)α-L-GalNAcA-1→</td>
<td>²H</td>
<td>5.11</td>
<td>4.29</td>
<td>4.15</td>
<td>4.21</td>
<td>4.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>¹³C</td>
<td>99.2</td>
<td>46.9</td>
<td>68.5</td>
<td>77.6</td>
<td>67.1</td>
<td>172.6</td>
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<tr>
<td>Gly</td>
<td>²H</td>
<td>173.5</td>
<td></td>
<td>3.92, 4.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>¹³C</td>
<td>43.2</td>
<td></td>
<td></td>
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</tbody>
</table>

Chemical shifts for NAc are: δH 1.97 and 2.06; δC 23.4, 23.6 (both CHO) and 175.0 (CO).


