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5-Acetamido-3,5-dideoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid-containing O-polysaccharide from marine bacterium *Pseudomonas glareae* KMM 9500^T

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ABSTRACT

The O-polysaccharide was isolated from the lipopolysaccharide of a marine bacterium *Pseudomonas glareae* KMM 9500^T and studied by chemical methods along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy including ^{1}H,^{1}H-TOCSY, ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-ROESY, ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC experiments. The O-polysaccharide was found to consist of linear tetrasaccharide repeating units constituted by D-glucuronic acid (D-GlcA), L-rhamnose (L-Rha), D-glucose (D-Glc) and 5-acetamido-7,9-*O*-[(*S*)-1-carboxyethylidene]-3,5-dideoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (Sug7,9(*S*-Pyr)), partially O-acetylated at position 8 (~70%):

→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-β-Sug*p*8Ac(~70%)7,9(*S*-Pyr)-(2→

1. Introduction

Bacteria of the genus *Pseudomonas* are ubiquitous microorganisms and are associated with a diverse range of environments including soil, plants, animals, clinical material, mineral waters as well as marine environments [1]. Most Gram-negative bacteria contain lipopolysaccharide (LPS) in their outer membrane. LPS is a heat-stable complex of amphiphilic macromolecules that provides an extraordinary permeability barrier to many different classes of molecules including detergents, antibiotics, and toxic dyes and metals. Moreover, the LPS is indispensable for viability and survival of Gram-negative bacteria, as it contributes to the correct assembly of the outer membrane. Owing to their external location, LPS molecules also interact with other biological systems by participating in host-bacterium interactions like adhesion, colonization, virulence, and symbiosis [2].

LPS can be classified as smooth (S-form) or rough (R-form) depending on their structural characteristics. Both types contain lipid A, covalently linked to a core oligosaccharidic region. Only in the S-form of LPS, the core region is substituted by an O-specific polysaccharide portion (OPS) [3]. The OPS of the marine Gram-negative bacteria LPS make up a group of polymers in which the structural variations are almost unlimited [4].

While many terrestrial isolates of the genus *Pseudomonas* have been investigated extensively, *Pseudomonas* strains from marine sources are still poorly studied. Recently, we showed that marine bacterium *P. xanthomarina* KMM 1447^T produced two types of LPS with different OPS [5]. One more study was devoted to the chemical and immunological characterization of marine-derived *P. stutzeri* KMM 226 LPS [6]. In present work, we continued the study of LPS from marine pseudomonads and established the structure of the OPS from *P. glareae* KMM 9500^T, a Gram-negative bacterium isolated from a sediment sample collected from the Sea of Japan seashore [7].

2. Results

LPS was isolated from dried bacterial cells by the phenol-water procedure and purified by an enzymatic treatment. SDS-PAGE

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analysis of the LPS gave a smooth-type LPS separation profile (Fig. 1) in which the ladder-like bands were typical of LPS having an O-polysaccharide with different average molecular weight.

The OPS was obtained by mild acid degradation of the LPS in acetate buffer. The sugar analysis using GC-MS of the acetylated methyl glycosides derived after methanolysis of the OPS revealed glucose (Glc), rhamnose (Rha) and glucuronic acid (GlcA). The absolute D configuration of Glc and GlcA, as well as the L configuration of Rha, were determined by GC of the acetylated (*S*)-2-octyl glycosides with appropriate standards. Further study of the OPS by NMR spectroscopy revealed the presence of one more component – 5-acetamido-7,9-*O*-[(*S*)-1-carboxyethylidene]-3,5-dideoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid (see below).

The ^{13}C (Fig. 2a) and ^{1}H NMR spectra of the OPS showed a structural heterogeneity owing to non-stoichiometric O-acetylation (there were signals at δ_C 21.9 and δ_H 2.17 for CH_3 of an O-acetyl group). The ^{13}C NMR spectrum of the O-deacetylated OPS (OPS-1, Fig. 2b) showed signals of five carbons in the anomeric region at δ 96.4–102.7. As judged by the data of the DEPT-135 experiment, the signals at δ 100.8 and 101.5 belonged to quaternary carbons. There were also signals of three carboxyl carbons at δ 175.8 and 177.0 (double intensity), two methyl carbons at δ 18.0 and 26.2, methylene carbon (data of the DEPT-135 experiment) at δ 36.2, nitrogen-bearing carbon at δ 47.1, two hydroxymethyl carbons (data of the DEPT-135 experiment) at δ 62.0 and 67.7, two carbons of an *N*-acetyl group at δ 23.6 (CH_3) and 176.9 (CO), and other carbons at δ 63.1–79.1. The total number of signals in the spectrum demonstrated the presence in the tetrasaccharide repeating unit of a nine-carbon sugar residue along with three hexose (D-GlcA, D-Glc, L-Rha) and pyruvic acid (Pyr) residues. The absence in the ^{13}C NMR spectrum of any signals for sugar ring carbons in the region δ_C 82–88 characteristic for furanosides demonstrated the pyranose form of all sugar residues.

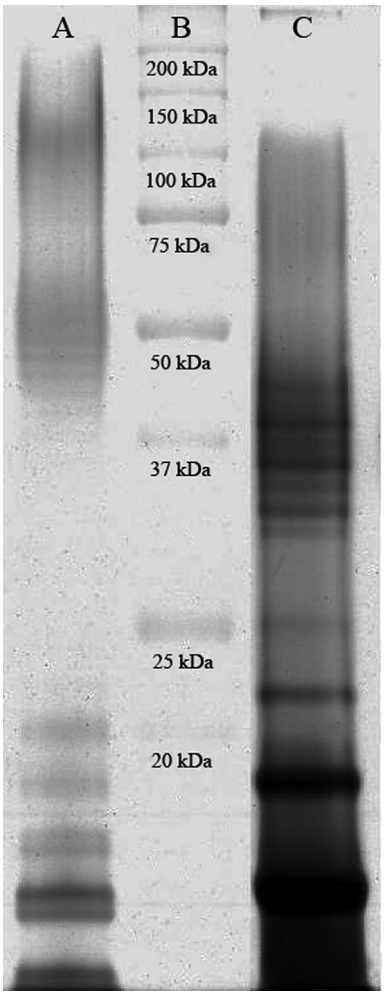

Fig. 1. – Silver-stained SDS-PAGE electrophoresis of *P. glareae* KMM 9500^T LPS. Lane A – *E. coli* 0111:B4 LPS 8 μg, lane B – unstained standard, lane C – *P. glareae* KMM 9500^T LPS 16 μg.

The ^{1}H NMR spectrum of the OPS-1 revealed, *inter alia,* characteristic signals for three protons in the anomeric region at δ 4.69 ($^3J_{1,2}$ 8.0 Hz), 4.85 and 5.07 ($^3J_{1,2}$ 3.8 Hz), one methylene group at δ 1.83 ($^3J_{3ax,4}$ 12.7 Hz) and 2.70, one *N*-acetyl group at δ 2.18 and two methyl groups at δ 1.33 ($^3J_{5,6}$ 5.9 Hz) and 1.41.

The ^{1}H and ^{13}C NMR spectra of the OPS-1 were assigned by 2D homonuclear ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-TOCSY, ^{1}H,^{1}H-ROESY, heteronuclear ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC experiments, and the chemical shifts for each sugar spin system are presented in Table 1.

The ^{1}H,^{1}H-COSY and ^{1}H,^{1}H-TOCSY spectra revealed proton spin systems for one sugar residue having the *manno* configuration (H-1/H-2 and H-2 up to H-6 correlations) and two having the *gluco* configuration (correlations between all ring proton). The sugar residue with the *manno* configuration was identified as L-Rha*p* (**R**) based on H-6/C-6 correlation at δ_H/δ_C 1.33/18.0 in the ^{1}H,^{13}C-HSQC spectrum. The sugar residues with the *gluco* configuration were identified as D-Glc*p* (**G**) and D-Glc*p*A (**GA**) based on H-6/C-6 and H-5/C-6 correlations at δ_H/δ_C 3.79, 3.93/62.0 and δ_H/δ_C 4.23/177.0in the ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC spectra (Fig. 3), respectively. The $^1J_{C1-H1}$ coupling constant values determined from the gated-decoupling spectrum of the OPS-1 showed that L-Rha*p* and D-Glc*p* were β-linked (164 and 166 Hz, respectively), whereas D-Glc*p*A was α-linked (171 Hz).

For the nine-carbon sugar, the ^{1}H, ^{1}H-COSY and ^{1}H, ^{1}H-TOCSY spectra correlated H-$3_{ax,eq}$/H-4,5 and H-6/H-7,8,$9_{ax,eq}$ protons. The ^{1}H,^{13}C-HSQC spectrum showed, *inter alia*, cross-peaks of H-5 with a nitrogen-bearing carbon at δ_H/δ_C 4.47/47.1and of H-$9_{ax,eq}$ with a substituted hydroxymethyl carbon at δ_H/δ_C 4.04, 3.92/67.7. The ^{1}H, ^{13}C-HMBC spectrum (Fig. 3, Table 2) indicated cross-peaks of H-3_{ax}/C-1 and H-3_{ax}/C-2 at δ_H/δ_C 1.83/175.8 and δ_H/δ_C 1.83/100.8, respectively, thus indicating the presence of a 5-acetamido-3,5-dideoxynon-2-ulosonic acid (**S**).

In addition, the ^{1}H, ^{13}C-HMBC spectrum (Fig. 3, Table 2) showed H-3/C-2 and H-3/C-1 correlations of Pyr at δ_H/δ_C 1.41/101.5 and δ_H/δ_C 1.41/177.0, respectively. The C-2 of Pyr showed correlation to H-$9_{ax,eq}$ of 5-acetamido-3,5-dideoxynon-2-ulosonic acid (**S**) at δ_C/δ_H 101.5/4.04 and 101.5/3.92, whereas H-3 of Pyr showed correlations to C-9 and C-7 of the higher sugar (**S**) at δ_H/δ_C 1.41/67.7 and δ_H/δ_C 1.41/72.4 [8], respectively, thus indicating a six-member dioxane ring of 5-acetamido-7,9-*O*-[1-carboxyethylidene]-3,5-dideoxynon-2-ulosonic acid (Fig. 3, Table 2). The absence of ^{1}H,^{1}H-ROESY correlations between H-3 of Pyr and any other protons was consistent with its equatorial orientation and *S*-configuration.

A $^3J_{3ax,4}$ coupling constant value of 12.7 Hz and a small $^3J_{4,5}$ (2.9 Hz) and $^3J_{5,6}$ (1.5 Hz) values indicated that H-4 of 5-acetamido-7,9-*O*-[(*S*)-1-carboxyethylidene]-3,5-dideoxynon-2-ulosonic acid occupied the axial position and H-5 the equatorial position, and, hence, the C-4,5,6 fragment had the *lyxo* configuration. A large $^3J_{6,7}$ value of 9.6 Hz showed the *erythro* configuration of the C-6,7 fragment [9]. Small $^3J_{8,9ax,eq}$ values (~2.1 Hz) indicated the *gauche* orientation of H-8 and H-$9_{ax,eq}$, which is preferable for the L-*glycero*-L-*manno*-configuration of 5-acetamido-7,9-*O*-[(*S*)-1-carboxyethylidene]-3,5-dideoxynon-2-ulosonic acid (otherwise the $^3J_{8,9ax}$ values would be ~12 Hz [10]). Moreover, the ^{1}H,^{1}H-ROESY spectrum showed an inner-residue H-8/H-6 cross-peak of 5–acetamido-7,9-*O*-[(*S*)-1-carboxyethylidene]-3,5-dideoxynon-2-