Carbohydrate Research 465 (2018) 40–43

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structure of the O-specific polysaccharide from *Azospirillum fermentarium* CC-LY743^T

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ARTICLE INFO

Keywords:
Lipopolysaccharide
Bacterial polysaccharide structure
Azospirillum fermentarium

ABSTRACT

O-specific polysaccharide was obtained by mild acid hydrolysis of the lipopolysaccharide of nitrogen-fixing bacterium *Azospirillum fermentarium* CC-LY743^T (IBPPM 578) and was studied by sugar analysis along with ^{1}H and ^{13}C NMR spectroscopy, including ^{1}H,^{1}H COSY, TOCSY, ROESY, and ^{1}H,^{13}C HSQC and HMBC experiments. The polysaccharide was found to be linear and to consist of alterating α-L-fucose and α-D-mannose residues in tetrasaccharide repeating units of the following structure: →2)-α-D-Man*p*-(1 → 3)-α-L-Fuc*p*-(1 → 3)-α-D-Man*p*-(1 → 3)-α-L-Fuc*p*-(1→

Currently, the genus *Azospirillum* of free-living alphaproteobacteria from the family Rhodospirillaceae comprises 19 species [1]. Many species of azospirilla are plant-growth-promoters, which have been isolated from the soil and the rhizosphere of wild grasses and agricultural crops [2,3]. High genomic plasticity and some physiological features of azospirilla, such as versatile C- and N-metabolism and the ability to accumulate polyhydroxyalkonates and undergo transition to cyst-like forms [2,3], make these bacteria adapted to environments other than those just mentioned. Several *Azospirillum* species have been isolated from non-plant-associated sources: oil-contaminated soil (*Azospirillum rugosum*) [4], discarded road tar (*Azospirillum picis*) [5], a sulphide spring (*Azospirillum thiophilum*) [6], a fermenter (*Azospirillum fermentarium*) [7], and a microbial fuel cell (*Azospirillum humicireducens*) [8].

Lipopolysaccharide (LPS) is a predominant component of the outer membrane of Gram-negative bacteria. Owing to its surface location, LPS is involved in interaction with environment [9]. The classical LPS (S-LPS) molecule has a tripartite structure and includes lipid A, core oligosaccharide and O-specific polysaccharide (OPS). OPS-free LPS is called R-LPS, or lipooligosaccharide.

The OPSs of *Azospirillum brasilense*, *Azospirillum halopraeferens*, and *Azospirillum lipoferum* have been studied in detail [10,11], whereas information on the OPS structures of the other *Azospirillum* species is absent. This work reports a structure of the OPS from the type strain of *Azospirillum fermentarium*.

The lipopolysaccharide of *A. fermentarium* CC-LY743^T was extracted from bacterial biomass by hot aqueous phenol. The SDS-PAGE analysis of the LPS (Fig. 1) showed the presence of fast-, and slow-migrating fractions in the regions corresponding to the R- and S-forms of *Pseudomonas putida* LPS, respectively. The predominance of S-LPS molecules evidenced the presence of high-molecular-mass OPS.

The LPS was degraded under mild acidic conditions. Lipid A sediment was removed by centrifugation, the OPS-containing supernatant was fractionated by GPC on Sephadex G-50. Fuc and Man were identified in an equal molar ratio in the OPS by the GLC of the alditol acetates. Determination of the absolute configuration revealed that Man has the D configuration and Fuc has the L configuration.

The structure of the OPS was studied in detail by NMR spectroscopy. The ^{1}H NMR spectrum of the OPS showed signals for four anomeric protons at δ 4.98–5.21, two CH_3–C groups (H-6 of Fuc) at δ 1.19 and other sugar protons at δ 3.78–4.38. The ^{13}C NMR spectrum of the OPS (Fig. 2) contained signals for four anomeric carbons at δ 97.4–102.9, two CH_3–C groups (C-6 of Fuc) at δ 16.9, two OCH_2–C group (C-6 of Man) at δ 61.9 and 62.4, and other sugar ring carbons in the region of δ 66.5–78.8. The absence of signals in the region of δ 83–88 that are characteristic of furanosides [12] confirmed the pyranosidic form of all monosaccharide residues.

The ^{1}H and ^{13}C NMR spectra of the OPS were assigned using 2D homonuclear ^{1}H, ^{1}H COSY, TOCSY, ROESY, and heteronuclear ^{1}H, ^{13}C HSQC, and HMBC experiments (Table 1, Supporting Information File). Based on intraresidue H,H and H,C correlations and $^3J_{H,H}$ coupling constant values, spin systems of four monosaccharide residues, two

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https://doi.org/10.1016/j.carres.2018.06.003
Received 11 April 2018; Received in revised form 28 May 2018; Accepted 5 June 2018
Available online 06 June 2018
0008-6215/

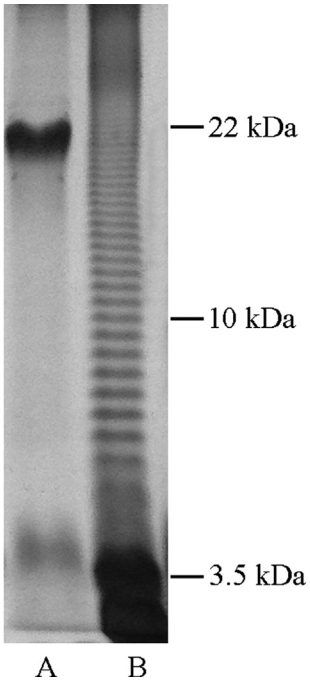

Fig. 1. Silver-stained SDS PAGE of the LPS from *A. fermentarium* CC-LY743^T Lane A – *A. fermentarium* CC-LY743^T LPS, 15 μg; Lane B – *P. putida* TSh-18 LPS 10 μg. M_w values were calculated based on the known structure of the *Pseudomonas* LPS.

having *manno* configuration (**A** and **C**) and two having *galacto* configuration (**B** and **D**), were identified. The TOCSY spectrum showed H-1/H-2, H-2/H-3,4,5,6 cross-peaks for Man residues, as well as H-1/H-2,3,4 and H-6/H-5 cross-peaks for Fuc residues. The signals within each spin system were assigned using the COSY spectrum.

The α configuration of Man and Fuc residues were inferred from relatively high-field positions of the C-5 signals at δ 74.6–74.7 and δ 68.2–68.3, respectively, in the ^{13}C NMR spectrum as compared with the published data of the corresponding α- and β-isomers [12,13].

The following interresidue correlations between anomeric protons and protons at the linkage carbons were present in the ROESY spectrum of the OPS: **A** H-1/**B** H-3 at δ 5.21/4.06; **B** H-1/**C** H-3 at δ 5.09/3.94, **C** H-1/**D** H-3 at δ 5.16/4.02, **D** H-1/**A** H-2 at δ 4.98/4.07. The ^{1}H, ^{13}C HMBC spectrum of the OPS contained the respective correlations between the following anomeric protons and transglycosidic carbons (Fig. 3): **A** H-1/**B** C-3 at δ 5.21/78.8; **B** H-1/**C** C-3 at δ 5.09/77.5, **C** H-1/**D** C-3 at δ 5.16/78.5, **D** H-1/**A** C-2 at δ 4.98/77.4. These data defined the glycosylation pattern and the monosaccharide sequence in the repeating unit. The positions of substitution of the monosaccharides were confirmed by significant downfield displacements of the C-3 signals of **B**, **C**, **D residues** and the C-2 signal of **A** residue as compared with their positions in corresponding unsubstituted monosaccharides [12,13].

Therefore, the OPS consists of linear tetrasaccharide repeating units (Chart 1). To our knowledge, this structure has not been found in bacterial polysaccharides earlier. Structurally similar exopolysaccharide was isolated from the culture medium of *Alcaligenes latus* [14] (currently *Azohydromonas lata*) and was found to have disaccharide repeating units were composed of 2-substituted Man and 3-substituted Fuc.

1. Experimental

1.1. Bacterial strain, growth, isolation and degradation of the lipopolysaccharides

Azospirillum fermentatium CC-LY743^T [7] (IBPPM 578) was obtained from the microbial culture collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov). The bacteria were cultivated at 30 °C in a liquid synthetic medium as described elsewhere [15] to late exponential phase, and cells were harvested by centrifugation. Capsular polysaccharides were removed by repeated washing with 0.15 M NaCl, cells were washed with acetone and dried on air. The biomass (10 g) was extracted by the Westphal procedure [16], proteins and nucleic acids were precipitated by CCl_3CO_2H (pH 2.7) and removed by centrifugation. After dialysis of the supernatant, LPS preparation was obtained in yield 9.7%.

LPS sample (111 mg) was hydrolysed with aq 2% AcOH at 100 °C for 3 h, the lipid precipitate was removed by centrifugation (13,000 × g, 20 min), and the carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 Superfine in 0.05 M pyridinium acetate buffer, pH 4.5. The elution was monitored by a Knauer differential refractometer. High-molecular-mass OPS preparation was

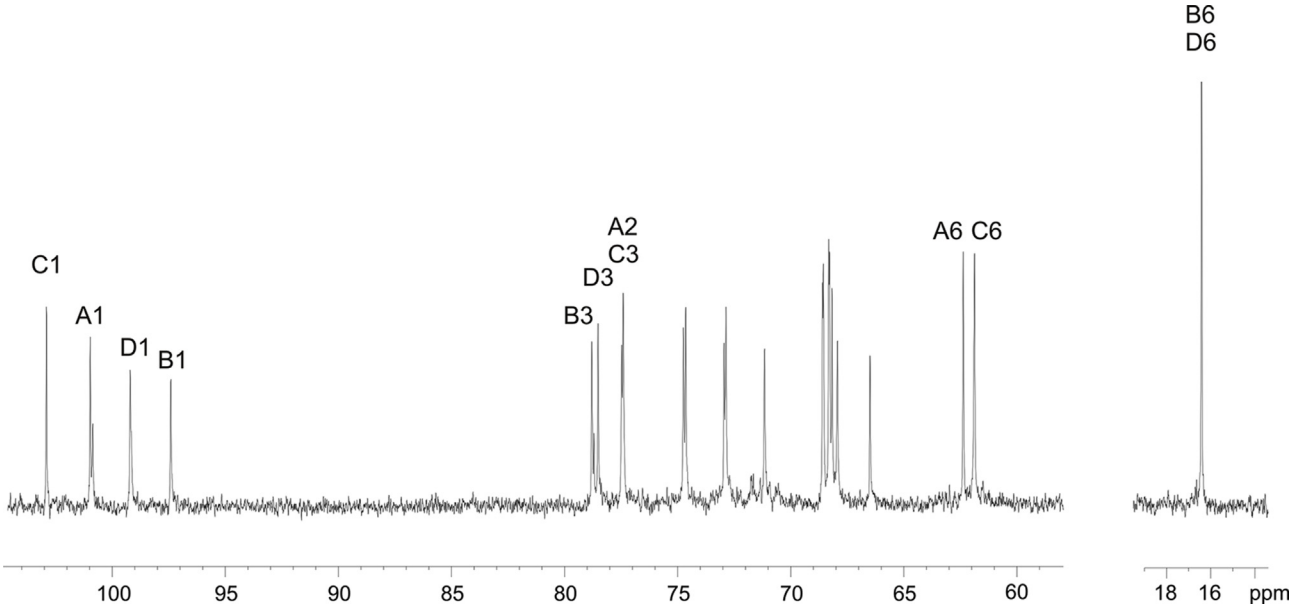

Fig. 2. ^{13}C NMR spectrum of the OPSs from *A. fermentarium*. Arabic numerals refer to protons in sugar residues denoted as follows: A, Man^I; B, Fuc^I; C, Man^{II}; D, Fuc^{II}.