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EXPERIMENTAL ARTICLES

Lipopolysaccharide of *Budvicia aquatica* 97U124: Immunochemical Properties and Structure

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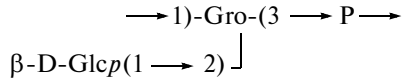
Abstract—The structure and immunochemical properties of lipopolysaccharide from *Budvicia aquatica* 97U124, a representative of a novel species of *Enterobacteriaceae*, were studied. The O-polysaccharide (OPS) was isolated by mild acid hydrolysis and characterized by monosaccharide analysis and NMR spectroscopy. It was shown that the OPS from *B. aquatica* 97U124 consisted of repeating units with the structure



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Keywords: Budvicia aquatica, lipopolysaccharide, O-specific polysaccharide, structure.

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The family *Enterobacteriaceae* is constantly replenished with new bacterial species, among which is the poorly studied species *Budvicia aquatica*, which was originally found in Europe. In 1983, Aldova et al. [1] isolated a novel group of hydrogen-sulfide-producing enterobacteria from drinking water in regions of the Czech Republic and assigned them to a new species *Budvicia aquatica*. Simultaneously, Richard et al. [2] isolated similar bacterial strains from river water in Switzerland and Spain and designated them as *"Citrobacter atypical."* The subsequent exchange of the data on the biochemical characteristics of these strains and with live cultures made it possible to conclude that they belonged to the same group of bacteria. Investigation of the genome of these bacteria (nucleotide composition and DNA–DNA hybridization) confirmed that all these isolates belonged to the same new genus (species) [3]; representatives of the species *Budvicia aquatica* were revealed by Pokhil in Ukraine [4].

Since the structure and composition of lipopolysaccharide (LPS), the major component of external membranes of gram-negative bacteria, are important chemotaxonomic criteria, the aim of this work was to isolate and characterize the LPS from a strain of *B. aquatica*. Until now, no data on the LPS of these bacterial group have been known.

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MATERIALS AND METHODS

The study was carried out with strain *Budvicia aquatica* LEPMD 97U124 isolated from a practically healthy person; it was obtained from the Collection of the Laboratory of Experimental and Applied Molecular Diagnostics, Mechnikov Institute of Microbiology and Immunology, Academy of Medical Sciences of Ukraine. Bacteria were cultivated in liquid N medium [5] on a shaker (220 rpm) at 28°C for 24 h.

Bacterial cells were collected by centrifugation (5000 *g*, 20 min), suspended in 100 ml of physiological saline solution, and repeatedly centrifuged. The washed cells were dehydrated with acetone (twice) and diethyl ether. The LPS was isolated from dried cells according to the method [6] by extraction with 45% solution of phenol in water at 65–68°C. After centrifugation of the cooled emulsion, three layers were formed (water, phenol, and precipitate); the upper water layer was removed; the phenol layer and precipitate were thoroughly mixed with distilled water (1 : 1). The water phases were combined and dialyzed against distilled water for 3–4 days to remove phenol and low-molecular impurities. The residue was concentrated under vacuum at 37°C. The LPS was purified from nucleic acids by triple ultracentrifugation (105000 *g*, 4 h); the precipitate was diluted with water and lyophilized.

After cleavage of the LPS with 3% acetic acid (100°C, 4 h), the sediment of lipid A was collected by centrifugation (13000 *g*, 20 min). To isolate the fractions of O-specific polysaccharide (OPS) and core oli-

gosaccharide, the supernatant was fractionated on a column (70 ± 3 cm) with Sephadex G-50 using 0.025 M pyridine–acetate buffer (pH 4.5) as the eluting agent.

Analyses of carbohydrates [7], protein [8], and nucleic acids [9] of LPS were performed. Heptose was determined by cysteine–sulfuric acid reaction [10]; 2-keto-3-deoxyoctonic acid (KDO) was assayed using thiobarbituric acid [10].

To analyze the fatty acid composition of LPS, the sample (10 mg) was dissolved in 3 ml of 1.5% solution of acetyl chloride in methanol and hydrolyzed in sealed ampoules (100°C, 4 h). Methyl esters of fatty acids were extracted three times with hexane (3 ml), vacuum-dried, and analyzed on an Agilent 6890/5973N chromatograph–mass spectrometer equipped with an HP-5MS column and a temperature program from 150 to 250°C at 4°C/min; helium was used as the carrier gas. The fatty acid methyl esters were identified by comparing their retention times with those of the standards (Supelco, United States).

To determine the monosaccharides, the preparations were hydrolyzed with 2 M HCl at 105°C for 5 h; after evaporation, the monosaccharides were reduced with sodium borohydride to acetate polyols, acetylated, and analyzed on an Agilent 6890N/5973-inert chromatograph–mass spectrometer equipped with a DB-225 mS column (30 m × 0.25 mm × 0.25μm); helium (1 ml/min) was used as the carrier gas; injector and interface were kept at 250 and 280°C, respectively; the column temperature in the isothermal condition was 220°C. The sample was injected with flow distribution of 1 : 100. In order to determine the composition of neutral monosaccharides, the preparations were hydrolyzed with 2 M CF_3CO_2H at 120°C for 2 h, and acetate polyols were analyzed on a Hewlett-Packard 5880 (United States) device equipped with an Ultra 2 capillary column; the column temperature was kept at 160°C for 1 min and then increased to 290°C at a rate of 7°C/min [11, 12]. Monosaccharides were identified by comparing retention times of acetate polyols with those of the standards and by using the ChemStation database.

The amino acids and amino sugars released after hydrolysis of the preparations with 6 M HC1 (100°C, 20 h) were assayed on a KLA-5 amino acid analyzer (Hitachi, Japan).

The absolute configurations of the monosaccharides were determined by GLC of acetylated glycosides with the use of (S)-2-octanol on a Hewlett-Packard 5880 chromatograph; the column temperature was kept at 160°C for 1 min and then increased to 290°C at a rate of 7°C/min [12].

The ^1H- and ^{13}C-NMR spectra of the preparations were recorded in D_2O at 27°C using a Bruker DRX-500 spectrometer (Germany), sodium-3-trimethylsilyl propanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.45) were used as internal standards, and the mixing time in the total correlation spectroscopy (TOCSY) was 200 ms.

O-antiserum was obtained by immunization of rabbits with 0.1–1.0 ml of a heat-treated (100°C, 2.5 h) cell suspension of *B. aquatica* 97U124 (2×10^9 cells/ml). Immunization was performed intravenously five times at 4-day intervals [10].

The antigenic activity of LPS was studied using double immunodiffusion in agar by the Ouchterlony method [13].

The pyrogenic properties of LPS were examined using 2.0- to 3.5-kg rabbits [10]. Thermometry of animals was performed by introducing an Omron electronic thermometer (Matsusaka, Japan) into the rectum at a depth of 5–7 cm, depending on the rabbit weight. The immune reactivity of all rabbits was preliminarily tested by intravenous injection of 0.9% sterile apyrogenic solution of sodium chloride (10 ml/kg). The LPS preparations were dissolved in sterile apyrogenic isotonic solution, incubated at 37°C for 10 min, and then injected intravenously (1 ml/kg). The minimal pyrogenic dose of the LPS preparations was determined by injection of their serial dilutions (from 0.5 to 1.0×10^{-2} mg/ml) into three rabbits differing in weight by less than 0.5 kg.

Prior to LPS injection, the temperature of the rabbits was measured twice at a 30-min interval (difference in the temperature values should not exceed 0.2°C); the value of the last measurement was taken as an initial temperature. The LPS solution was injected within 15–20 min after the last temperature test. The subsequent temperature measurements were performed three times at 1-h intervals. The LPS preparation was considered apyrogenic if the total increase in the temperature of three rabbits did not exceed 1.4°C.

The toxicity of the LPS was studied with the use of healthy white mice of both sexes weighing 19–21 g, which were not earlier used in experiments. All mice were sensitized by intraperitoneal injection of 0.5 ml of 3.2% solution of D-galactosamine hydrochloride in apyrogenic sterile 0.9% NaCl solution; immediately after that, 0.2 ml of the LPS solution in sterile isotonic physiological saline (preliminary heated to 37°C) was injected intraperitoneally at a rate of 0.1 ml/s. The toxicity (LD_{50}) of the LPS preparations was determined using ten mice for each set of serial dilutions; in the control (ten mice), intraperitoneal introduction of sterile 0.9% solution of NaCl (0.2 ml) together with D-galactosamine hydrochloride was performed. The animals were observed for 48 h [10].

RESULTS AND DISCUSSION

The lipopolysaccharide extracted from dry bacterial cells with a water–phenol mixture was characterized by a high content of nucleic acids (19.5%). Purification of the LPS preparation from nucleic acids involved several cycles of ultracentrifugation that

Table 1. Monosaccharide composition of the LPS and its components from *B. aquatica* LEPMD 97U124

Monosaccharides	LPS	OPS (I)	Core (II)	Core (III)
	% of the sum of peak areas			
X_1			17.5	4.4
Glucose	93.4	79.2	51.0	95.6
Galactose	4.8	11.6	16.2	
Rhamnose	1.8	9.2	15.3	
	Weight % of dry preparation			
Geptose	3.6		3.4	2.0
Glucosamine	2.5	Traces	1.75	0.25
KDO	Traces		0.12	0.03

Table 2. Fatty acid composition of the LPS from *B. aquatica* LEPMD 97U124

Acid	% of the sum of peak areas
Dodecanoic $C_{12:0}$	3.6
X*	1.1
Tetradecanoic $C_{14:0}$	15.1
Iso-pentadecanoic *i*-$C_{15:0}$	9.0
Anteiso-pentadecanoic *ai*-$C_{15:0}$	14.0
3-Hydroxytetradecanoic $3OHC_{14:0}$	**21.4**
Iso-hexadecanoic i-$C_{16:0}$	2.0
Hexadecenoic $C_{16:1}$	6.6
Hexadecanoic $C_{16:0}$	**18.6**
Octadecanoic $C_{18:0}$	1.1

Note: X* stands for an unidentified derivative with a retention time of 10.88 min.

decreased the LPS yield to 3.4% of dry cells. The purified LPS preparation contained carbohydrates (38%), nucleic acids (2.2%), and protein (3.5%).

Monosaccharide analysis of the LPS from *B. aquatica* LEPMD 97U124 showed that glucose prevailed (86.9%); rhamnose, galactose, and heptose amounted to 1.1, 5.3, and 6.7%, respectively; glucosamine comprised 2.5% of dry weight; and KDO was revealed in trace amounts (Table 1).

The LPS from *B. aquatica* contained fatty acids with a chain length from 12 to 18 carbon atoms (Table 2). In lipid A, 3-hydroxytetradecanoic and hexadecanoic acids prevailed (21.4 and 18.6%, respectively); tetradecanoic, *anteiso*-pentadecanoic, and *iso*-pentadecanoic acids comprised 15.1, 14.0, and 9.0%, respectively; the amounts of other acids varied from 1.1 to 3.6%. Unsaturated hexadecenoic acid (6.6%) was also revealed in the LPS from *B. aquatica*; its formation is considered to be due to the dehydration of hydroxy acids [14].

The structural components of LPS were isolated after mild acid hydrolysis of the preparations, the precipitate of lipid A was removed by centrifugation, and water-soluble carbohydrates were separated by gel filtration on Sephadex G-50 (Fig. 1). It was shown that the LPS contained the mixture of S- and R-forms of molecules that is demonstrated by the presence of a high-molecular OPS (fraction I) and of low-molecular weight core fractions (fractions II and III).

The core was an acid oligosaccharide directly linked to lipid A; it was found in LPSs of all the studied gram-negative bacteria. Fraction II of the core was shown to contain neutral monosaccharides: rhamnose, glucose, and galactose (34.5, 28.3, and 19.7%, respectively); unidentified sugar (17.4%); and heptose (3.4%) (Table 1).

Only D-isomers of monosaccharides were revealed in the OPS; glucose prevailed (79.2%); galactose and rhamnose comprised 11.6 and 9.2%, respectively (Table 1).

The ^{13}C NMR spectrum of OPS displayed the signals of one anomeric carbon atom of glucose at 103.7 ppm, one hydroxymethyl group (C-6 Glc) at 62.1 ppm, and signals of the remaining carbon atoms of the monosaccharide cycle in the region of 71.0–77.3 ppm (Fig. 2). The signals of substituted C-1, C-3, and C-2 atoms of glycerol at 66.3, 66.5, and 79.0 ppm, respectively, were also revealed in the spectrum. Accordingly, the ^1H-NMR spectrum of OPS exhibited the signals of one anomeric proton at 4.67 ppm and the remaining protons at 3.33–4.21 ppm. The absence of signals in the region of 83–88 ppm in the ^{13}C NMR spectrum indicated that the glucose residue was in pyranose form [15].

Comparing the ^{13}C NMR spectrum of OPS with those of 2-substituted β-glucosyl residues of glycerol teichoic acids from the cell walls of *Arthrobacter crystallopoietes* [16], *Clavibacter michiganensis* [17], and *Nocardiopsis tregalosei* [18] showed that the repeating unit of OPS from *B. aquatica* 97U124 had the following structure:

$$\begin{array}{l} \longrightarrow 1)\text{-Gro-}(3 \longrightarrow \text{P} \longrightarrow \\ \beta\text{-D-Glc}p(1 \longrightarrow 2)\rfloor \end{array}$$

Similar structures are typical of teichoic acids of gram-positive bacteria and rarely occur in gram-negative bacteria.

The residues of galactose and rhamnose revealed in small amounts in OPS were probably components of the core oligosaccharide of this LPS.

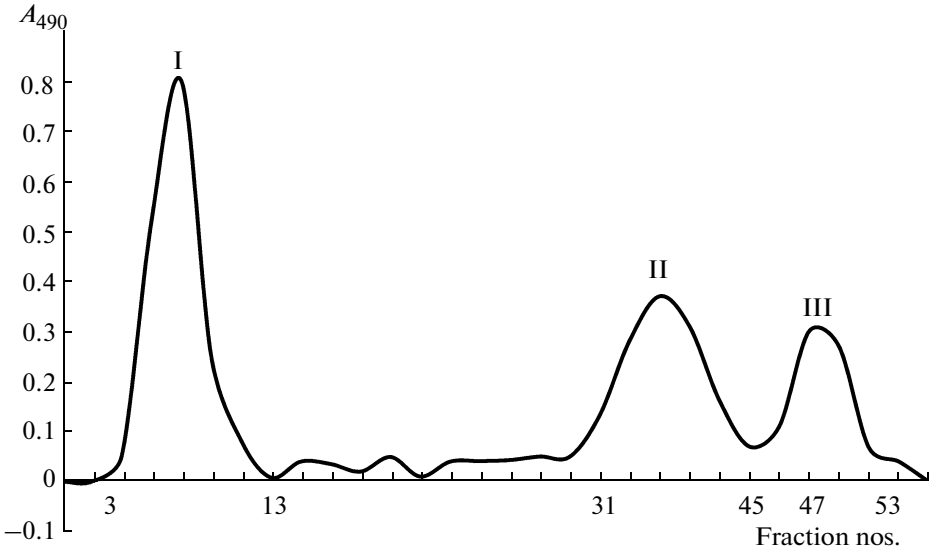

Fig. 1. Elution profile of the carbohydrate moiety of degraded LPS from *B. aquatica* LEPMD 97U124 obtained on a Sephadex G-50 column.

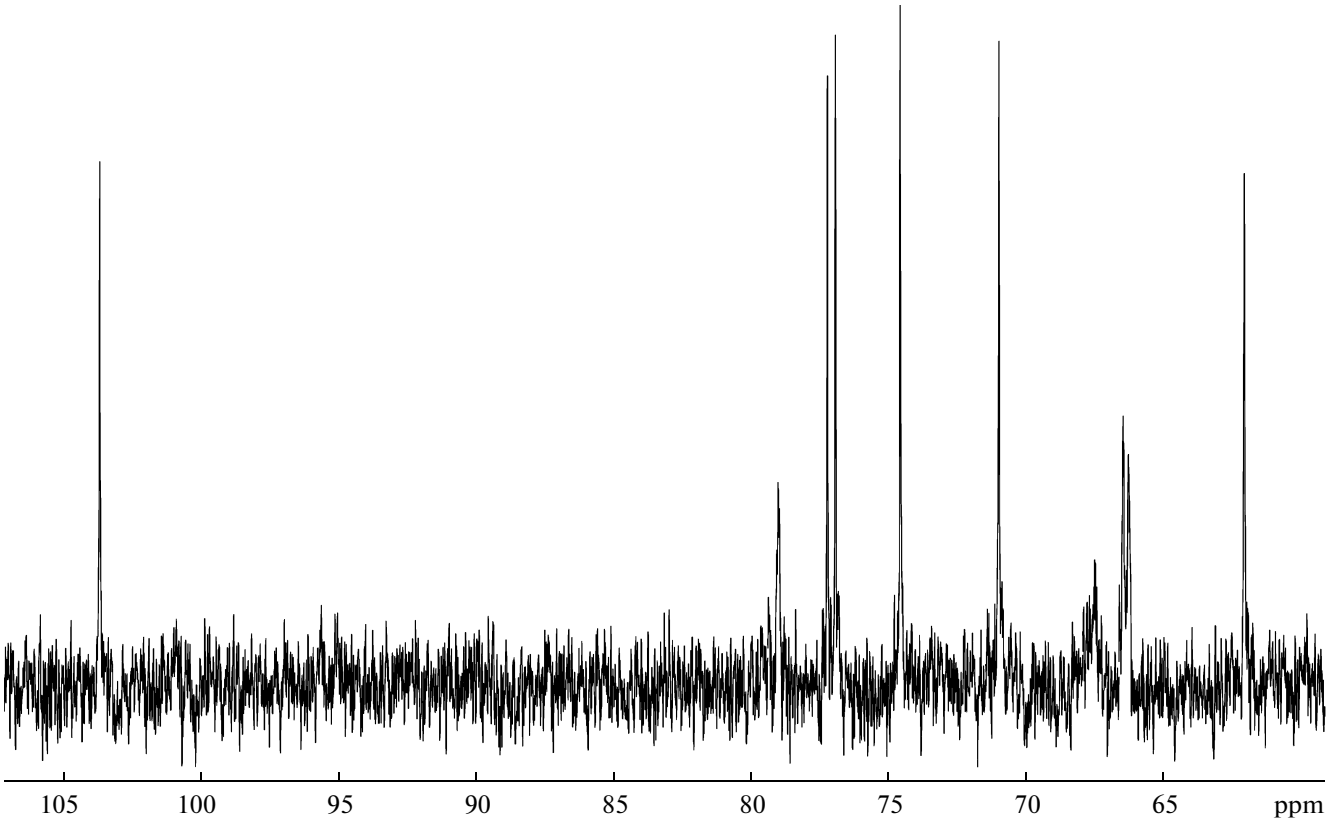

Fig. 2. The ^{13}C-NMR spectrum of the OPS from *B. aquatica* LEPMD 97U124.

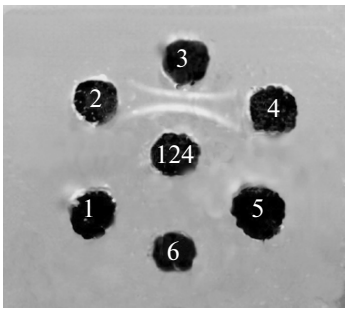

Fig. 3. Reaction of double immunodiffusion in agar gel of the LPSs from *B. aquatica* strains: LEPMD 97U101 (*1*), LEPMD 97U126 (*2*), LEPMD 97U124 (*3*), DRL 20186 (*4*), DRL 23270 (*5*), and DRL 24833 (*6*) performed with O-antiserum to strain *B. aquatica* LEPMD 97U124 by the Ouchterlony method.

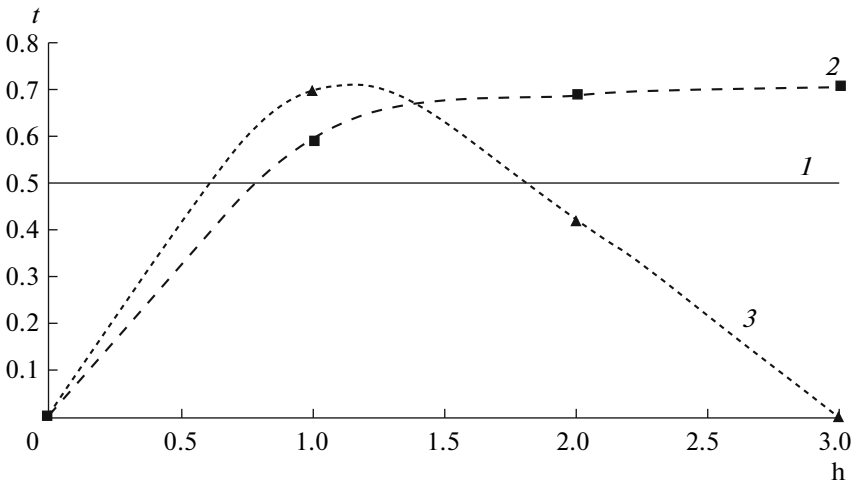

Fig. 4. Pyrogenic activity of the LPS from *B. aquatica* LEPMD 97U124. Pyrogenal (*1*), pyrogenicity threshold (*2*), and *B. aquatica* (*3*).

Since LPS is the main thermostable antigen of microbial cells, its composition and structure determine the serological O-specificity of bacteria. In serological studies, rabbit polyclonal antisera obtained to heat-treated *B. aquatica* 97U124 culture were used as antibodies; the LPS isolated from this strain served as an antigen. The antiserum titer in the ring precipitation reaction was 1 : 20000. In reactions of double immunodiffusion carried out according to the Ouchterlony method, the LPS from *B. aquatica* 97U124 showed antigenic activity in a homologous system. In cross-serological reactions between the LPSs from strains of *B. aquatica* DRL 20186, DRL 23270, and DRL 24833, the antiserum obtained to *B. aquatica* 97U124 did not react with any of the studied LPSs that may indicate that common antigenic

Table 3. Data of 500-MHz ^{1}H NMR and 125-MHz ^{13}C NMR spectra of the OPS from *B. aquatica* LEPMD 97U124 (ppm)

Residue	H-1 (1a/1b) C-1	H-2 C-2	H-3 (3a/3b) C-3	H-4 C-4	H-5 C-5	H-6a/6b C-6
β-D-Glc*p*-(1 →	4.67 103.7	3.33 74.6	3.52 77.3	3.40 71.0	3.49 77.0	3.92/3.74 62.1
→ 2,3)-Gro-(1 →	3.90/3.98 66.5	4.21 79.0	4.06/4.10 66.3			

determinants are absent and the studied strains belong to different serological groups (Fig. 3).

It was shown that the LPS from *B. aquatica* 97U124 exhibited low toxicity and was apyrogenic (Fig. 4).

Thus, for the first time, the lipopolysaccharide was isolated from strain *B. aquatica* 97U124, a representative of a novel *Enterobacteriaceae* species, with the OPS containing glucose and glycerol phosphate and a structure unusual for gram-negative bacteria. The OPS is structurally similar to glycerol teichoic acids of gram-positive bacteria; like this class of polymers, LPS from *B. aquatica* 97U124 showed low toxicity and was apyrogenic.

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