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EXPERIMENTAL ARTICLES

Characterization of the Lipopolysaccharide from *Budvicia aquatica* DLR 20186

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Abstract—A lipopolysaccharide (LPS) from *Budvicia aquatica* DRL 20186 was isolated, studied, and chemically identified. It was shown to be lowly toxic, but highly pyrogenic. Its fatty acid composition was similar to that of the LPS from other *Enterobacteriaceae*, with predominance of tetradecanoic (32.7%) and 3-hydroxytetradecanoic acids (23.8%). Hexadecenoic (20.4%), hexadecanoic (11.8%), and dodecanoic acids (8.4%) were also revealed. Double immunodiffusion in agar by the Ouchterlony method revealed antigenic activity of the *B. aquatica* DLR 20186 LPS in a homologous system. In cross reactions, however, it did not interact with the antisera of other *B. aquatica* strains.

Keywords: *Budvicia aquatica*, lipopolysaccharide, fatty acid composition, monosaccharide composition, biological activity

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Enterobacteria have been among the first studied microorganisms, since they are an indispensable part of the biosphere and are extremely abundant in various environments. The activity of these bacteria considerably affects diverse aspects of human life. In spite of intensive phenotypic and genetic studies of enterobacteria, the great variety of their biological properties complicates their diagnostics; therefore, taxonomy of the family *Enterobacteriaceae* is continuously changed and improved. The major problem is the identification of novel (newly described) and poorly studied species, such as *Rahnella aquatilis, Budvicia aquatica*, and *Pragia fontium*, which exhibit low affinity to the representatives of the main genus, *Escherichia* (16, 5, and 1%, respectively). The genome structure of the genus *Pragia* is the most similar to that of *Budvicia* (20–37% of the DNA–DNA homology). The genus *Budvicia* with only one species *B. aquatica* was first described in 1983 in Czechoslovakia [1]. These bacteria were found in various water sources (wells, water ducts, streams, rivers, and swimming pools) [2].

At present, it is considered that virtually all members of the family *Enterobacteriaceae* can be infectious under certain conditions. Out of novel species, only *R. aquatilis* strains were shown to be associated with lesions of the urinary tracts, gastrointestinal tract, respiratory apparatus, pro-inflammatory wound infections, and abscesses of various localization; they were isolated from patients afflicted with diabetes, bronchial asthma, and emphysema. An opinion exists that *B. aquatica* strains are unable to cause infectious diseases since there are no conditions for their development in a human organism; they may, however, be involved in enteric infections of small mammals and in plant diseases. However, there is information that *B. aquatica* was found in blood and urine of a sepsis-suffering 85-year-old woman, a denizen of New Orleans, who was a victim of Hurricane Katrina in 2007; this bacterium was also revealed in the water flooding her house.

Since lipopolysaccharides (LPS) are known as the key components responsible for the pathogenicity of gram-negative bacteria, the goal of the present work was to isolate and identify the LPS from the type strain of *B. aquatica* and to study its functions and biological activity.

MATERIALS AND METHODS

Subject of investigation. The study was carried out with strain *B. aquatica* DRL 20186 obtained from the Culture Collection of the Diagnostic and Research Laboratory, Budapest, Hungary, which was isolated from well water (Prague Institute of Hygiene and Epidemiology, Czech Republic). Batch cultivation of bacteria was performed in the synthetic medium N [4] on a shaker at 28–30°C for 24 h. Bacterial cells were harvested by centrifugation (5000 *g*, 20 min) and dried with acetone and diethyl ether.

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Isolation of LPS. The LPS was extracted from dried cells with phenol (45% aquatic solution) at 65–68°C. The obtained aquatic fractions were dialyzed sequentially against tap and distilled water for phenol removal [5].

Assays of carbohydrates, nucleic acids, and protein. The concentration of carbohydrates was determined by their reaction with phenol and sulfuric acid; the color of the reaction product was measured on a spectrophotometer at 490 nm; the calibration curve was constructed with glucose as the standard [6].

Nucleic acids were assayed by the Spirin method [7]; the amount of protein was determined using Folin reagent by the Lowry method [8].

Identification of neutral monosaccharides was carried out after hydrolysis of the preparations with 2 M HCl (100°C for 5 h). Monosaccharides were analyzed in the form of polyol acetates [9] on an Agilent 6890/5973N chromatograph–mass spectrometer (United States) equipped with a DB-225mS column (30 m × 0.25 mm × 0.25 μm); the carrier gas was helium at a flow rate of 1 mL/min; the temperatures of evaporator and interface were 250 and 280°C, respectively; column temperature was 220°C (isothermal regime). Monosaccharides were identified by the retention times of their polyol acetates compared with those of the standards and by means of the ChemStation database. The amounts of individual monosaccharides were expressed as percentages either of the sum of the peak areas or of the dry preparation weight.

The absolute configurations of monosaccharides were determined by gas–liquid chromatography (GLC) of acetylated glycosides with the use of (S)-2-octanol on a Hewlett-Packard 5880 chromatograph (United States) at a programmed temperature gradient from 160 (1 min) to 290°C at a rate of 7°C/min [10].

Determination of the fatty acid composition. To analyze the fatty acid composition of the LPS, the preparations were hydrolyzed with a 1.5% solution of acetyl chloride in methanol (100°C for 4 h), and fatty acid methyl esters were analyzed on an Agilent 6890N/5973 inert chromatograph–mass spectrometer (United States) equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 μm) at the temperature programmed to rise from 150 to 250°C at 4°C/min; the carrier gas was helium at a flow rate of 1.2 mL/min; the temperature of evaporation was 250°C; the flow distribution was 1 : 100. Identification of fatty acids was performed with the use of the database of a personal computer as well as of the standard mixture of the fatty acid methyl esters.

NMR spectroscopy. The NMR spectra of the O-specific polysaccharide (OPS) preparations were recorded in 99.95% D_2O at 30°C on a Bruker Avance II 600 spectrometer (Germany) with the use of 3-trimethylsilylpropanoate-2,2,3,3-d_4 (δ_H 0.00 ppm) and acetone (δ_C 31.45 ppm) as internal standards. The samples were lyophilized from 99.5% D_2O prior to analysis.

Pyrogenicity of the LPS. The pyrogenic properties of the LPS preparations were examined using 2.0- to 3.5-kg rabbits [11]. Thermometry of the 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 preliminary 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 the 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 the initial temperature. The LPS solution was injected within 15–20 min after the last temperature measurement. The subsequent temperature measurements after injection were performed three times at 1-h intervals. The LPS preparation was considered apyrogenic if the total increase in the temperature for 3 h did not exceed 1.4°C.

Determination of the LPS toxicity. The toxicity of LPS was studied with the use of healthy mongrel white mice of both sexes weighing 19–21 g, which have not been used in earlier 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 (preheated to 37°C) was injected intraperitoneally at a rate of 0.1 mL/s. The dose of the LPS preparation causing the death of 50% of the test animals (LD_{50}) 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 [11].

Immunological studies. The O-antiserum was obtained by five intravenous immunizations of rabbits at 4-day intervals with 0.1 to 1.0 mL of heat-treated (100°C, 2.5 h) cell suspension of *B. aquatica* (2 × 10^9 cells/mL) [11].

The antigenic activity of LPS was studied using double immunodiffusion in agar by the Ouchterlony method [12].

Statistical analysis. The results were statistically processed according to the method offered for the pharmaceutical preparations [11].

Table 1. Fatty acid composition of the LPS from *B. aquatica* DRL 20186

Fatty acid	% of the sum of peak areas
$C_{12:0}$	8.4
$C_{14:0}$	32.7
$aiC_{15:0}$	1.2
3-OH-$C_{14:0}$	23.8
$C_{16:1}$	20.4
$C_{16:0}$	11.8
cis-9,10-$C_{17:0}$	1.7

RESULTS AND DISCUSSION

The LPS extracted from the *B. aquatica* DRL 20186 cells with a water–phenol mixture was characterized by a high content of nucleic acids (32.5%), which could be expected when taking into account the method of its extraction. Purification of the LPS preparation from nucleic acids involving three cycles of ultracentrifugation decreased the nucleic acid content to 9.4%. The yield of purified LPS from *B. aquatica* DRL 20186 (4.0%) was lower than those from the other representatives of gram-negative bacteria, e.g., *Escherichia coli* (about 5%), and the previously studied novel enterobacteria species, such as *R. aquatilis* [13–17] and *P. fontium* [18, 19] (21.7 and 19.8%, respectively).

The purified LPS preparation from *B. aquatica* DRL 20186 contained rather low amounts of carbohydrates (28.0%) and trace amounts of protein. The observed low content of carbohydrates was probably due to the presence of hexosamines, which produce no color reaction with the phenol and sulfuric acid used for determination of the total carbohydrates.

The monosaccharide analysis of the LPS preparation showed that galactose (39.8%), glucose (25.0%), and rhamnose (18.0%) prevailed; ribose and glucosamine amounted to 0.9 and 1.35%, respectively. The preparation also contained the typical LPS components, such as heptoses (16.3%) and 2-keto-3-deoxyoctulosonic acid (KDO) (0.09%).

Thus, the LPS from the cell wall of the studied *B. aquatica* strain contained all components typical of this polymer.

Lipopolysaccharides of gram-negative bacteria are known as the key components responsible for pathogenicity. The LPSs are in close contact with the membrane proteins and assure integrity, stability, and functioning of the outer cell membranes; they have an important role in the interactions of bacterial cells with the environment or with the host organisms (in the case of pathogenic microorganisms) as O-antigens and endotoxins. Many consequences of bacterial infections, e.g., endotoxemia and bacterial shock, are associated with unique endotoxic properties of the LPS, among which toxicity and the ability to activate the cells of the host immune system are the most important. Specific interaction of LPS with the cells of a macroorganism results in the synthesis of active mediators–cytokines, which at low concentrations regulates the functioning of the immune system of the organism, whereas at high concentrations it causes a complex of toxic effects including pyrogenicity, leukopenia, and septic shock. In an LPS molecule, lipid A is known to act as the endotoxic center, with the properties depending on the amount of fatty acids, the level of phosphorylation, the presence of substitutes at C4', and specific configuration [20–25].

According to the results of chromatography–mass spectrometry analysis of the fatty acid methyl esters of the LPS from *B. aquatica*, lipid A contained fatty acids with chain length from C_{12} to C_{17} (Table 1). The predominant fatty acids included tetradecanoic (32.7%), 3-hydroxytetradecanoic (23.8%), and hexadecenoic (20.4%) acids. Since lipid A is a highly conservative part of the LPS molecule, its fatty acid composition may be used as an additional chemotaxonomic criterion for the species identification. The members of the family *Enterobacteriaceae* are characterized by the presence of only 3-hydroxytetradecanoic fatty acid, which acylates both amino and hydroxyl groups of the glucosamine residues in lipid A [25]. Our results represent additional evidence for assignation of the studied strain to enterobacteria. For most studied strains of gram-negative bacteria, fatty acid composition of lipid A is a species-specific criterion, whereas for the members of *Enterobacteriaceae*, it is a common characteristic of the family.

Lipid A, its structure, biosynthesis, modification, and composition of its individual components attract increasing interest of researchers, since it is responsible for most biological activities of the LPS molecules. It is known that LPSs exhibit mitogenic, antimetastatic, antileucosis, radioprotector, immunomodulating, and antitumor activities, as well as induce syntheses of the tumor necrosis factor, interleukine-1, and γ-interferon [26]. However, practical application of LPSs as therapeutic preparations is hindered by their high toxicity, pyrogenicity, and other harmful properties.

The toxicity of LPS preparations was estimated using the LD_{50} index characterizing a dose of the preparation which caused death of 50% of the experimental animals. It was found that LD_{50} of the LPS from *B. aquatica* DRL 20186 was 100 μg/mouse (Table 2). It can be noted that toxicity of the LPS from *B. aquatica* DRL 20186 was lower than that for other members of the family *Enterobacteriaceae*, in particular, *R. aquatilis, P. fontium*, and *E. coli* [13, 15].

The minimal pyrogenic dose of the LPS from *B. aquatica* was 7.5×10^{-3} μg/mL of apyrogenic isotonic solution. It was revealed that the temperature

Table 2. Biological activity of the LPS from *B. aquatica* DRL 20186

Strain	Minimal pyrogenic dose for rabbits, μg/mL	Average temperature shift (°C) after LPS injection			LD_{50}	
		1 h	2 h	3 h	μg/mouse	mg/kg
DRL 20186	0.0075	+1.4	+0.8	+0.8	100.0	5.0

shift caused by the LPS solutions was over +0.45°C, which exceeded the physiological standard for healthy animals (Fig. 1, Table 2). The temperature of experimental animals sharply increased in an hour after the LPS injection, slightly decreased after two hours and then normalized. It was shown that pyrogenic activity of the LPS from *B. aquatica* was higher than that of pyrogenal, the pharmaceutical preparation based on the *Shigella typhi* LPS.

The LPSs are the main thermostable antigens of microbial cells; their composition and structure determine the serological specificity of bacteria. In serological studies, the rabbit polyclonal antisera obtained by the heat-treated *B. aquatica* culture were used as antibodies; the LPS isolated from the studied 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* showed antigenic activity in a homologous system. In serological cross reactions between the LPSs from *B. aquatica* strains DRL 23270, DRL 24833, LNMIZ 96U101, 97U124, and 97U126, the antiserum to *B. aquatica* DRL 20186 did not react with any of the studied LPSs, probably indicating the absence of common antigenic determinants in these strains (Fig. 2). Thus, serological studies indicated immunochemical heterogeneity of the species *B. aquatica*.

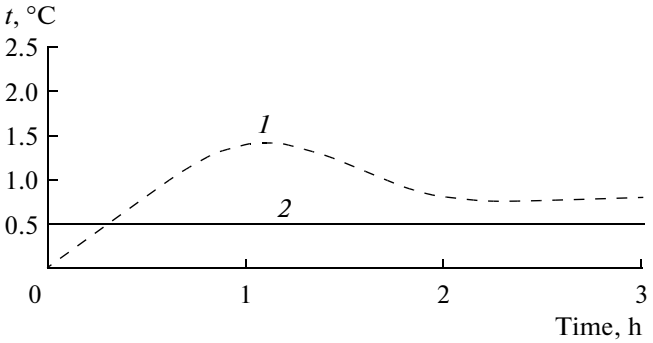

Fig. 1. Pyrogenic activity of the LPS from *B. aquatica* DRL 20186 (*1*) and the pyrogenicity threshold (*2*).

We have earlier established the structure of the O-specific polysaccharide (OPS) from strain *B. aquatica* 97U124: it consisted of repeating units of glycerol teichoic acid with β-D-glucopyranose at the second carbon atom [26, 27]. To study the structure of the OPS from *B. aquatica* DRL 20186, the OPS isolated by mild acid hydrolysis of the LPS was analyzed by gel chromatography on a column with Sephadex G-50.

The structure of the OPS was established by monosaccharide analysis, determination of the absolute configurations, as well as by using 1D and 2D ^{1}H and ^{13}C NMR spectroscopy (Table 3). It was shown that the OPS from *B. aquatica* DRL 20186 had the following structure [28]:

$$\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1 \rightarrow 2)\text{-}$$

$$\alpha\text{-Yer}p\text{-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow$$

A specific feature of this polysaccharide is the presence of an uncommon branched monosaccharide, yersiniose A. This monosaccharide or its (*R*)-1'-hydroxyethyl isomer (yersiniose B) have been revealed earlier in the LPSs from various species of *Yersinia*

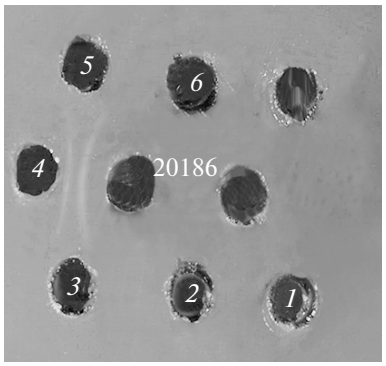

Fig. 2. Reaction of double immunodiffusion in agar for the LPSs from *B. aquatica* LNMIZ 96U101 (*1*); *B. aquatica* LNMIZ 97U126 (*2*), *B. aquatica* LNMIZ 97U124 (*3*), *B. aquatica* DRL 20186 (*4*), *B. aquatica* DRL 23270 (*5*), and *B. aquatica* DRL 24833 (*6*) with O-antiserum to *B. aquatica* DRL 20186 carried out by the Ouchterlony method.

Table 3. Data on chemical shifts (δ, ppm) in the OPS from *Budvicia aquatica* DRL 20186

Monosaccharide residue	H-1	H-2	H-3 (3eq, 3ax)	H-4	H-5	H-6 (6a, 6b)	H-1'	H-2'
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*
→3)-β-D-Gal*p*NAc-(1→	4.82	4.02	3.87	4.20	3.67	3.85, 3.79		2.04
	102.8	*52.5*	*76.9*	*65.1*	*75.7*	*62.2*	*175.8*	*23.6*
→4)-α-L-Rha*p*-(1→	5.00	4.02	3.96	3.68	3.85	1.35		
	103.3	*71.5*	*71.5*	*81.3*	*68.8*	*18.3*		
→3)-α-D-Gal*p*-(1→	5.15	3.94	3.90	4.05	4.02	3.74		
	99.5	*68.5*	*78.7*	*70.4*	*72.8*	*62.6*		
→2)-α-Yer*p*-(1→	5.15	4.11	1.94, 2.00		4.26	1.15	3.70	1.18
	93.2	*72.1*	*29.8*	*76.3*	*68.8*	*13.5*	*70.6*	*16.6*

[29], *Burkholderia brasilensis* [30], and *Legionella* species [31].

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