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EXPERIMENTAL ARTICLES

Characterization of the *Pragia fontium* Lipopolysaccharides

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Abstract—Lipopolysaccharides (LPSs) of two strains *Pragia fontium* 97U116 and 27480 were isolated and characterized; they were close to those of other representatives of the family *Enterobacteriaceae* in fatty acid composition and contained, respectively, 3-hydroxytetradecanoic acid as the predominant component (45.8 and 45.1%), tetradecanoic (23.5 and 28.9%), hexadecanoic (12.6 and 7.9%), hexadecenoic (12.6 and 7.9%), and dodecanoic (4.9 and 4.2%) fatty acids. The O-specific polysaccharides consisted of linear penta- and tetrasaccharide repeating units:
→2)-α-D-Gal*f*-(1→3)-α-L-Rha*p*2Ac-(1→4)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→
→4)-β-D-Man*p*NAc3NAcA-(1→2)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→
The LPSs of *P. fontium* 97U116 and 27480 were serologically active and belonged to different serogroups; they were less toxic than those of strain *E. coli* O55:B5, but more pyrogenic than the Pyrogenal preparation.

Keywords: lipopolysaccharide, *Pragia fontium,* structure, biological activity

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Although representatives of the family *Enterobacteriaceae* are well studied, both phenotypically and genotypically, their taxonomy is constantly being changed and improved. In recent years, several new genera characterized by different affinity to the type genus *Escherichia* have been described with the use of traditional and modern methods of chemo- and genosystematics. One of them, the genus *Pragia*, is represented by the species *Pragia fontium*. In 1988, Aldova et al. [1], based on analysis of the phenotypic and genotypic characteristics of 18 strains of hydrogen sulfide-producing atypical enterobacteria, concluded that they belonged to a novel genus of the family *Enterobacteriaceae* and were characterized by a low affinity to *Escherichia* (the DNA–DNA homology was 5%). In recent years, bacteria *P. fontium* have attracted increased attention of researchers due to their role in matter turnover in nature and the involvement in biocenoses of various ecological systems (soil, water, ponds, etc.). Bacteria *P. fontium* were also isolated from foodstuff, drinking water, and clinical samples taken from healthy persons and patients (excrements, pharynx- and purulent wound discharge, etc.).

Components of bacterial cell surface are known to be involved in the processes determining the biological features of microorganisms and interrelations between microorganisms, as well as between micro- and macroorganisms in biocenoses. Lipopolysaccharides (LPSs) are of special interest as the main components of outer membranes in gram-negative bacteria. LPSs exhibit a wide range of biological activities including endotoxic, in particular, pyrogenic reactions, lethal toxicity, Shwartzman reaction, etc. Being located on the cell surface, the LPSs interact with other biopolymers, stabilizing the outer membrane, carry out the barrier function preventing penetration of toxins, detergents, antibiotics, and other medical preparations into microbial cells, and serve as receptors of bacteriocins and phages. As the main thermostable antigens and endotoxins of microbial cells, the LPSs determine their serologic specificity and O-antigenicity, and participate in infectious processes. LPSs are known as transforming agents and nonspecific stimulators of defense reactions in macroorganisms. The studies of LPS properties are important for the development of scientific approaches aimed at the production of drugs, vaccines, and diagnostic tests, as well as for the biotechnologies for production of uncommon carbohydrates. The composition and structure of LPSs are recognized as important chemotaxonomic criteria in microbial taxonomy; individual components of the LPS molecule (lipid A, O-specific polysaccharide (O-PS), and core oligosaccharide) are characterized by different rates of evolutionary variability and thus can be used to characterize the taxa of different levels. Until now, no information was known concerning isolation, characterization, and biological activity of the

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LPS from *P. fontium*. However, such studies can gain insight into the mechanisms responsible for the development and induction of biological activity of LPSs and their functioning at molecular level. Information on the LPS structure is a basis for development of intraspecies classification systems of pathogenic bacteria and for identification of infective strains.

The aim of this work was to study the composition, structure, and some biological properties of the lipopolysaccharides from two strains of *Pragia fontium*.

MATERIALS AND METHODS

The study was carried out with strains *P. fontium* LEPMD 97U116 and DRL 27480 isolated from Vorskla River (Poltava oblast, Ukraine) and tap water (Vlachovo Brezi, Czech Republic), respectively. Bacteria were grown in flasks with synthetic medium N [2] on a shaker (220 rpm) at 28°C for 24 h; the cells were collected by centrifugation (5000 *g* for 30 min) and dehydrated by treatment with acetone and diethyl ether. Lipopolysaccharides (LPSs) were extracted from the cells with a water–phenol mixture at 65–68°C [3]. Nucleic acids were removed by precipitation with trichloroacetic acid and subsequent ultracentrifugation (104000 *g* for 4 h); the purified LPS preparation was lyophilized.

In order to obtain the fractions of O-PS and core oligosaccharide, the LPS preparation was hydrolyzed with 2% acetic acid (100°C for 3 h), the precipitate of lipid A was removed by ultracentrifugation (25000 *g* for 40 min), and then the supernatant was concentrated to 10 mL and 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.

The amounts of the following components were determined: nucleic acids (by the Spirin method [4]), carbohydrates (by reaction with phenol and sulfuric acid [5]), protein (by the Lowry method [6]), heptoses (by reaction with cysteine and sulfuric acid [7]), 2-keto-3-deoxyoctulosonic acid (KDO) (by reaction with thiobarbituric acid [8]).

Total phosphorus was analyzed by the Fiske–Subbarow method [9]. The LPS solution (1 mg/mL) was heated with concentrated sulfuric acid; then the mixture was cooled, supplemented with 2–3 drops of hydrogen peroxide and heated again for 20–30 min; successive addition of hydrogen peroxide and heating were repeated until complete decoloration of the solution. A sample of the colorless solution (0.5 mL) was neutralized with NaOH in the presence of phenolphthalein (1–2 drops), diluted with distilled water to 2.5 mL, supplemented with 1.5 mL of the molybdate reagent and vigorously stirred. A working solution of eikogen (1 mL) was added to all of the samples, the solutions were stirred, incubated at room temperature for 30–40 min and then cooled; the amount of phosphorus was measured spectrophotometrically at 625 nm using a calibration curve constructed on the basis of the standard KH_2PO_4 solution.

The amino acids and amino sugars released after hydrolysis of the preparations with 6 N HC1 (100°C for 20 h) were assayed on a KLA-5 amino acid analyzer (Hitachi, Japan). In order to determine the composition of neutral monosaccharides, the preparations were hydrolyzed with 2 N HCl (105°C for 5 h) or with 2 M CF_3CO_2H (120°C for 2 h) and analyzed in the form of acetate polyols on an Agilent 6890N/5973 inert chromatograph–mass spectrometer (United States) equipped with a DB-225 mS 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; the column temperature was 220°C (isothermal regime). The sample was injected into the column with flow distribution of 1 : 100. Acetate polyols were also analyzed on a Hewlett-Packard 5880 chromatograph (United States) equipped with an Ultra 2 capillary column using a programmed temperature range from 180°C (1 min) to 290°C at a rate of 10°C/min [10, 11]. Monosaccharides were identified from the retention times of their acetate polyols compared with those of the standards using the ChemStation database.

The absolute configurations of monosaccharides were determined by gas–liquid chromatography (GLC) of acetylated glycosides with (S)-2-octanol on a Hewlett-Packard 5880 chromatograph (United States) at 230°C for 20 min [12].

The polysaccharide was methylated by treatment with CH_3I in dimethyl sulfoxide in the presence of methylsulfinyl methanide [13] and then hydrolyzed by treatment with 2 M CF_3CO_2H (100°C for 2 h). The partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–mass spectrometry on a Hewlett-Packard 5880 chromatograph (United States) equipped with an HP-5ms column; the column temperature was kept at 150°C for 3 min and then raised to 320°C at a rate of 5°C/min.

The ^1H and ^{13}C NMR spectra of the preparations were recorded in D_2O at 27°C on a Bruker DRX-500 spectrometer (Germany). The chemical shifts were evaluated with the use of sodium-3-trimethylsilyl propanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.45) as internal standards. Times of 200 and 150 ms were used in the total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY), respectively.

To determine 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 methyl esters were analyzed on an Agilent 6890N/5973 inert chromatograph–mass spectrometer (United States) equipped with a computer.

Antisera were obtained by immunization of 2.5-kg rabbits with increasing doses of the heat-treated (100°C for 2.5 h) cell suspension of *Pragia fontium*

Table 1. Chemical composition of the *P. fontium* LPSs purified by ultracentrifugation (% of dry preparation)

Strain	Carbohydrates	Protein	Nucleic acids
97U116	55.1 ± 2.1	0.5 ± 0.2	4.9 ± 0.7
27480	42.2 ± 1.6	0.5 ± 0.1	1.1 ± 0.9

Table 2. The amounts of KDO, heptoses, and glucosamine in the LPSs from *P. fontium* (% of dry preparation)

Strain	KDO	Heptoses	Glucosamine
97U116	0.48 ± 0.05	4.9 ± 0.8	18.85
27480	0.31 ± 0.04	7.9 ± 0.8	17.66

(from 2×10^6 to 5×10^7 cells/mL). Immunization was performed four times at 7-day intervals. Blood samples were taken 7 days after the last injection. Double diffusion in agar gel was carried out according to the Ouchterlony method in 1% agarose gel using the physiological saline solution with 2% polyethylene glycol 6000 [14]. Imunoelectrophoresis was carried out in 1% agarose gel using 0.05 M veronal–medinal buffer (pH 8.6) at a current strength of 80–95 V (12 mA). After electrophoresis, the native antiserum was placed into gel trenches, incubated overnight at 37°C, and precipitation lines were observed.

Toxicity (LD_{50}) of the LPS was studied on mongrel white mice sensitized with galactosamine.

The pyrogenic properties of the LPS were examined by an intravenous injection of a predetermined minimal pyrogenic dose of the LPS into rabbits and subsequent thermometry of the animals for 3 h.

RESULTS AND DISCUSSION

The LPS content of *P. fontium* LEPMD 97U116 and DRL 27480 was 17.9 and 9.7%, respectively; this was higher than the average values determined for other representatives of *Enterobacteriaceae* (5%), but lower than in some strains of the genus *Pseudomonas* (up to 32%).

Because of the LPS extraction procedure applied, the preparations contained considerable amounts of nucleic acids (up to 30%), which were removed by ultracentrifugation. The purified preparations from strains *P. fontium* 97U116 and 27489 contained considerable amounts of carbohydrates (55.1 and 42.2%, respectively), insignificant quantity of nucleic acids (4.9 and 1.1%, respectively), and traces of protein (0.5%) (Table 1).

The monosaccharide fractions of the LPSs from both strains were composed of rhamnose, galactose, glucose, and heptoses (Tables 2 and 3). In the LPSs of *P. fontium* 97U116 and 27489, the amount of KDO, a typical LPS component in gram-negative bacteria, was insignificant (0.48 and 0.31%, respectively); glucosamine comprised 18.85 and 17.66%, respectively. According to the literature data, glucosamine was revealed in most of the lipid A samples studied. At the same time, it is known (together with other hexosamines) as a structural component of the polysaccharide moiety of the LPS molecule [15].

Thus, the isolated glycopolymers contained all the components typical of the LPSs.

In order to isolate the individual structural components of the LPS molecule, we applied mild acid hydrolysis, which cleaves the ketoside bond between the KDO residue and glucosamine II residue in the lipid A molecule. In the course of evolution, the individual components of the LPS macromolecule (lipid A, core oligosaccharide, and O-specific polysaccharide) showed different levels of variability. Lipid A is the most conservative moiety of the LPS molecule, as is indicated by the fact that it contains the antigenic epitopes responsible for cross reactions between remotely related taxa. Lipids A from various bacterial species are known to differ in the composition of fatty acids, which is a relatively stable characteristic and may be used as a chemotaxonomic criterion for elucidation of the phylogenetic relationship between microorganisms. Hydroxy acids are the most diagnostically important since they are bonded directly with the carbohydrate moiety of lipid A. It was shown that strains *P. fontium* 97U116 and 27480 con-

Table 3. Monosaccharide composition of the LPSs and core oligosaccharides from *P. fontium* (% of the sum of peak areas)

Strain	Preparation	Rhamnose	Ribose	Galactose	Glucose	Heptose
97U116	LPS	35.1 ± 1.7	–	31.2 ± 1.4	28.8 ± 1.1	4.9 ± 0.7
	Core oligosaccharide 1	4.05 ± 0.24	–	6.57 ± 0.39	45.98 ± 1.71	43.39 ± 1.56
	Core oligosaccharide 2	4.61 ± 0.26	20.65 ± 1.16	18.07 ± 1.01	41.9 ± 2.35	14.77 ± 0.83
27480	LPS	45.2 ± 2.0	–	18.6 ± 2.1	27.9 ± 1.9	8.3 ± 0.92
	Core oligosaccharide 1	14.64 ± 0.56	–	13.04 ± 0.5	42.06 ± 1.6	30.26 ± 1.15
	Core oligosaccharide 2	–	46.63 ± 1.91	8.11 ± 0.33	40.42 ± 1.66	4.84 ± 0.2

Note: "–" stands for "not detected".

Table 4. Fatty acid composition of lipid A from the *P. fontium* LPS (% of the sum of peak areas)

Strain	$C_{12:0}$	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$	Minor components
97U116	4.9 ± 0.2	23.5 ± 0.4	45.8 ± 0.4	8.6 ± 0.4	12.6 ± 0.1	4.6 ± 0.3
27480	4.2 ± 0.3	28.9 ± 0.1	45.1 ± 0.3	7.5 ± 0.2	7.9 ± 0.4	6.4 ± 0.5

tained fatty acids with a chain length from 10 to 16 carbon atoms: 3-hydroxytetradecanoic (the predominant component), tetradecanoic, hexadecanoic, hexadecenoic, and dodecanoic fatty acids (Table 4). Fatty acid composition of lipid A as the most conservative moiety of the LPS molecule can be used as an additional taxonomic criterion for species differentiation. Indeed, *P. fontium*, like other representatives of *Enterobacteriaceae*, contained the lipids with only one characteristic acid, 3-hydroxytetradecanoic acid, which is known to acetylate both amino and hydroxy groups of glucosamine. Thus, the obtained data confirm the classification of the studied strains as enterobacteria.

Lipids A of some bacteria may contain substituents, which change the biological properties not only of lipid A, but also of the whole bacterial cell. To reveal such substituents, in particular, 4-amino-4-deoxy-L-arabinose, an indirect method was used. The presence of such group in the lipid A molecule is known to make the cell resistant to some polycationic antibiotics, particularly to polymyxin B [16]. Since the studied strains *P. fontium* 97U116 and 27480 were sensitive to polymyxin B (the growth inhibition zones were 17 and 19 cm, respectively), it may be assumed that their lipid A does not contain 4-amino-4-deoxy-L-arabinose, and, therefore, polymyxin B can be attached to the glucosamine II molecule.

Lipid A is known as an endotoxic center of the LPS molecule, which determines such LPS properties as lethal toxicity, pyrogenicity, Schwartzman reaction, the ability to induce the tumor necrosis factor, and mutagenic activity. The toxicity of the LPSs from *P. fontium* 97U116 and 27480 were compared with that of the LPS from *E. coli* 055:B5 which is a classical endotoxin, usually applied as a control. The lethal toxicity (LD_{50}) of the LPSs from the studied strains determined in experiments with D-galactosamine-sensitized mice was 350 to 210 times lower than that of the *E. coli* LPS (Table 5). Therefore, the LPSs from *P. fontium* strains can be considered substances of low toxicity, with the LPS from *P. fontium* 97U116 1.7-fold less toxic than that from *P. fontium* 27480. The lipid A structure, in particular, the level of phosphorylation and fatty acid composition, are known to determine the LPS endotoxic activity. The LPSs from *P. fontium* 97U116 and 27480 had compatible fatty acid composition; the amounts of tetradecanoic (23.5 and 28.9%, respectively) and hexadecanoic (12.6 and 7.9%, respectively) acids, as well as phosphorus content (0.33 and 0.38%, respectively) differed insignificantly. It may be assumed that in the studied strains (a) lipid A fractions contain unidentified components which are responsible for the different levels of LPS toxicity; (b) LPSs are characterized by different ratios of hydrophilic and hydrophobic moieties; (c) the O-PSs have different structures.

Table 5. Toxicity of the LPS preparations from *P. fontium*

Strain	LD_{50}	
	μg/mouse	μg/kg
97U116	50	3333
27480	30	1875
E. coli O55:B5	0.14	8.0

Comparative studies showed that minimal pyrogenic dose of the LPSs from *P. fontium* was 7.5×10^{-3} μg/mL of apyrogenic isotonic solution. The LPSs from both strains caused an increase in the temperature of experimental animals by more than 0.45°C within the first two hours after injection, which exceeded the physiological standard for healthy animals (Table 6). The pyrogenic activity of the LPSs from the studied strains was more pronounced than that of the pharmaceutical preparation Pyrogenal containing the *Shigella typhi* LPS.

Thus, the LPSs of *P. fontium* 97U116 and 27480 were less toxic than those of *E coli* but more pyrogenic than the Pyrogenal preparation.

The structure of the core oligosaccharides, which was elucidated only recently, is known to be more variable than lipid A. Two low-molecular-weight fractions of core oligosaccharides eluted during gel filtration of the LPSs from *P. fontium* 97U116 and 27480 (Fig. 1) were heterogeneous in monosaccharide composition (Tables 3 and 7). In the core oligosaccharide fractions 1 and 2 from *P. fontium* 97U116 and in fraction 1 from *P. fontium* 27480, glucose and heptose prevailed, whereas in fraction 2 from *P. fontium* 27480, glucose and ribose predominated. Core oligosaccharides of the LPSs from the studied strains contained glucosamine (9.27 to 20.78%) and small amounts of KDO (0.48 to 0.60%).

The results on the heterogeneity of core oligosaccharides from the *P. fontium* LPSs are in agreement with the literature data, although this LPS moiety had been earlier considered as a slightly variable component.

Table 6. Pyrogenic activity of LPSs from *P. fontium*

Strain	Average values of temperature change (°C) after injection for		
	1 h	2 h	3 h
97U116	+0.5 ± 0.1	+0.9 ± 0.1	+1.2 ± 0.2
27480	+0.9 ± 0.2	+1.2 ± 0.1	+1.5 ± 0.1
Pyrogenal	+0.6 ± 0.1	+0.7 ± 0.1	+0.7 ± 0.1

The O-PS is the most variable component of the LPS molecule; in the studied strains, it was represented by two high-molecular-weight fractions (Fig. 1).

Analysis of the monosaccharide composition of O-PS from *P. fontium* 27480 showed the presence of glucose, rhamnose, and GlcNAc. Further studies revealed residues of 2,3-diamino-2,3-dideoxymannuronic acid (ManNAc3NAcA), which was not detected by monosaccharide analysis. The absolute configuration of rhamnose residues determined by GLC of (*S*)-2-octylglycosides showed that rhamnose had the L configuration. The GlcNAc and ManNAc3NAcA residues had the D configuration, as was determined on the basis of the glycosylation effects in the ^{13}C NMR-spectra of O-PS [17].

The ^{1}H-NMR spectrum of the O-PS displayed the signals of four anomeric protons at 4.84–5.17 ppm, two CH_3–C groups (two H-6 Rha residues) at 1.24 and 1.30 ppm, and other protons at 3.30–4.42 ppm, as well as the signals of *N*-acetyl groups at 1.89–2.07 ppm (Table 8).

The ^{13}C-NMR spectrum of the O-PS exhibited the signals of four anomeric carbon atoms at 98.0–102.6 ppm, three carbon atoms bonded with nitrogen atoms at 52.9–54.9 ppm, two CH_3–C groups (C-6 Rha) at 18.1 and 18.2 ppm, one $HOCH_2$–C group (C-6 GlcNAc) at 61.6 ppm, carbon atoms bonded with the oxygen atom in the monosaccharide cycle at 70–82 ppm, three *N*-acetyl groups at 23.3–23.4 ppm (CH_3), and CO NAc and CO_2H (C-6 ManNAc3NAcA) at 175.2–176.9 ppm (Table 8, Fig. 2). Based on the absence of signals at 83–88 ppm in the ^{13}C-NMR spectrum, it was concluded that all monosaccharides were in the pyranose form [18].

In the ^{1}H- and ^{13}C-NMR spectra of the preparations, the chemical shifts and spin systems of four monosaccharides were assigned with the use of 2D ^{1}H, ^{1}H COSY, TOCSY, ROESY, ^{1}H, ^{13}C heteronuclear HMBC and HSQC experiments and determination of the interaction constants ($^{3}J_{H,\,H}$) [18]. It was determined [19] that the O-PS from *P. fontium* 27480 consisted of a regularly repeating tetrasaccharide unit with the following structure:

→4)-β-D-Man*p*NAc3NAcA-(1→2)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→

This structure is unique among bacterial polysaccharides.

The O-PS of *P. fontium* 96U116 contained residues of galactose and rhamnose in a ratio of 1 : 3.5 and 2-acetamido-2-dideoxyglucose (GlcNAc). The L configuration of rhamnose residues and the D configuration of the Gal- and GlcNAc residues were determined by GLC of acetylated (*S*)-2-octylglycosides of the relevant sugars as well as on the basis of ^{13}C NMR spectra, taking into account the glycosylation effect [17].

The ^{13}C-NMR spectrum of the O-PS (Table 9, Fig. 3) displayed the signals of five anomeric carbon atoms at 96.5–103.0 ppm, two CH_3–C groups (C-6 Rha) at 17.9 and 18.0 ppm, two signals of carbon atoms bonded to nitrogen atoms at 55.6 and 57.1 ppm (C-2 GlcNAc), three signals of hydroxymethyl groups (C-6 Gal and GlcNAc) at 61.0–64.2 ppm, and other signals of carbon atoms of the monosaccharide cycle in the region of 70.3–84.8 ppm, as well as the signals of two *N*-acetyl groups at 23.3 and 23.6 ppm (both CH_3), 175.9 and 176.1 ppm (both CO), and the signals of one *O*-acetyl group at 22.1 ppm (CH_3) and 174.9 ppm (CO). The ^{1}H-NMR spectrum (Table 9) contained six signals in the region of 4.58–5.20 ppm for five anomeric protons and H-2 of 2-*O*-acetylated Rha, two signals of CH_3–C groups (H-6 Rha) at 1.27 and 1.28 ppm, signals of other protons of the monosaccharide cycle at 3.33–4.08 ppm, two *N*-acetyl groups at 2.06 and 2.07 ppm, and one *O*-acetyl group at 2.26 ppm.

Table 7. The amounts of KDO, heptoses, and glucosamine in core oligosaccharides of the LPSs from *P. fontium* (% of dry preparation)

Strain	Preparation	KDO	Heptoses	Glucosamine
97U116	Core oligosaccharide 1	0.54 ± 0.06	34.7 ± 2.1	16.9
	Core oligosaccharide 2	0.6 ± 0.05	12.8 ± 1.2	20.78
27480	Core oligosaccharide 1	0.52 ± 0.05	24.6 ± 1.5	9.27
	Core oligosaccharide 2	0.48 ± 0.04	6 ± 0.5	16.24

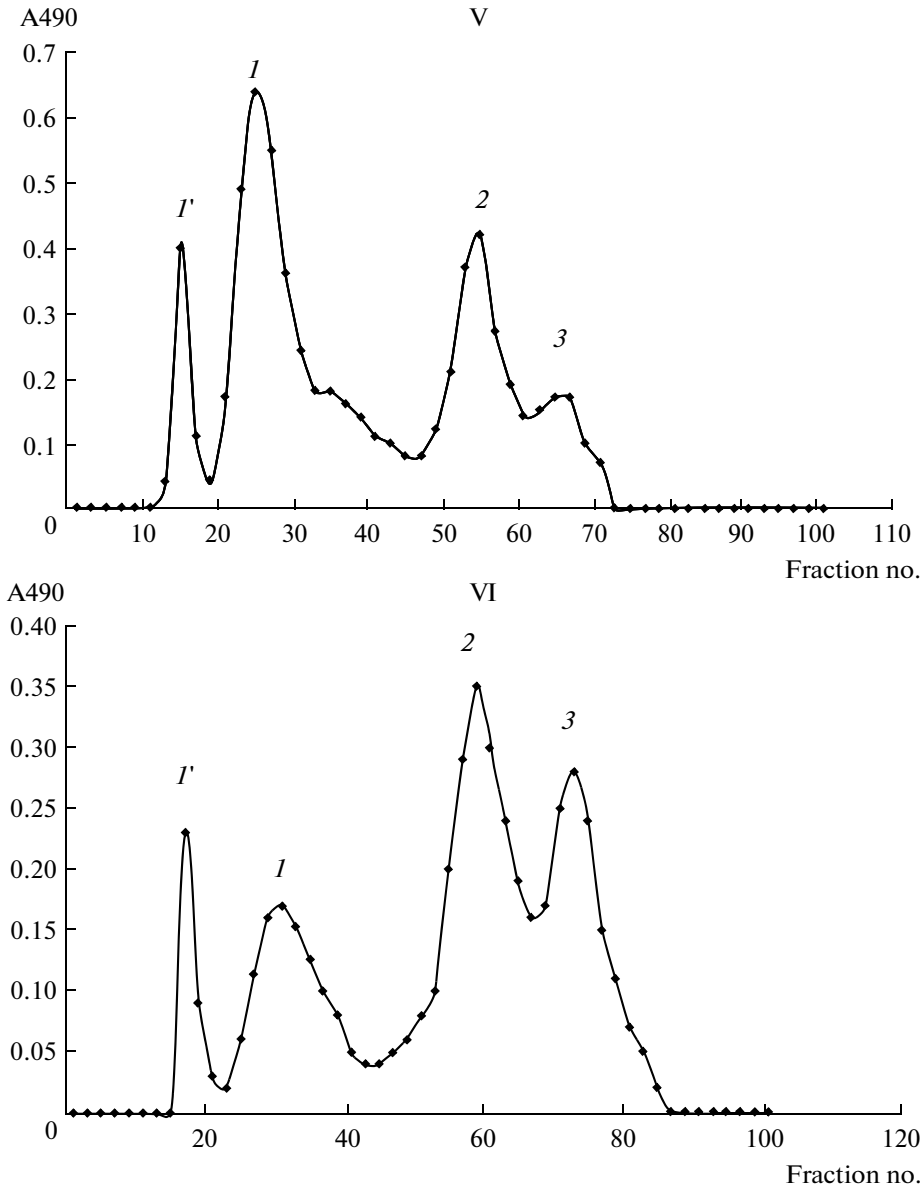

Fig. 1. Elution profiles from a Sephadex G-50 column of carbohydrate moiety of degraded LPS molecule from *P. fontium* 97U116 (V) and 27480 (VI). (*1*') and (*1*) designate the O-PS fractions; (*2*) and (*3*) indicate the core oligosaccharide fractions.

The spin systems of the monosaccharide residues were assigned using the combination of 2D ^{1}H, ^{1}H COSY, TOCSY, ROESY, ^{1}H, ^{13}C-heteronuclear HSQC, and HMBC experiments; the spin–spin interaction constants ($^{3}J_{H, H}$) were calculated from the 2D NMR spectra. The methods of determining the O-PS structure was described in detail elsewhere [20].

Thus, the O-PS of *P. fontium* 97U116 was shown to have the following structure:

→2)-α-D-Gal*f*-(1→3)-α-L-Rha*p*2Ac-(1→4)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→

The structure of the LPS, in particular, that of the O-PS, determines the serological specificity of microbial cells. Immunochemical properties of the LPSs were studied with the use of-polyclonal O-antisera obtained by rabbit immunization with heated cells of the studied *P. fontium* strains as controls. The titers of O-antisera determined by the ring precipitation and agglutination reactions were 2.5×10^{-5} and 1 : 6400 for strain 97U116 and 5.0×10^{-5} and 1 : 800 for strain 27480, respectively. By using the reactions of ring precipitation, agglutination, immunoelectrophoresis, and double immunodiffusion in agar gel by the Ouchterlony method, it was shown that the studied LPSs exhibited serological activities in homologous

Table 8. The ^{1}H- and ^{13}C-NMR data on chemical shifts in the O-polysaccharide from *P. fontium* 27480 (ppm)

Monosaccharide residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 (6a, 6b) C-6
→4)-β-D-Man*p*NAc3NAcA-(1→	4.99	4.42	4.26	3.93	3.96	
	101.9	52.9	54.7	71.6	78.8	175.6
→2)-α-L-Rha*p*-(1→	5.11	4.12	3.88	3.30	3.79	1.24
	102.6	80.3	71.1	73.7	70.5	18.2
→3)-β-L-Rha*p*-(1→	4.84	4.11	3.57	3.41	3.40	1.30
	102.0	71.9	81.5	72.8	73.4	18.1
→4)-α-D-Glc*p*NAc-(1→	5.17	3.84	3.78	3.66	3.68	3.72, 3.93
	98.0	54.9	71.6	77.9	72.2	61.6

Table 9. The ^{1}H- and ^{13}C-NMR data on chemical shifts in the O-polysaccharide from *P. fontium* 97U116 (ppm)

Monosaccharide residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a, 6b C-6
→2)-α-D-Gal*f*-(1→	5.20	4.08	4.37	3.87	3.77	3.62
	102.5	84.8	72.7	81.5	71.4	64.2
→3)-α-L-Rha*p*2Ac-(1→	4.99	5.09	3.94	3.60	4.12	1.28
	98.6	72.7	78.0	71.9	70.3	18.0[a]
→4)-α-D-Glc*p*NAc-(1→	4.86	3.92	3.89	3.68	4.08	3.84, 3.88
	96.5	55.6	70.5	78.4	72.5	61.0
→2)-α-L-Rha*p*-(1→	4.85	3.79	3.88	3.52	4.00	1.27
	99.4	77.1	70.8	73.2	70.7	18.0[a]
→3)-β-D-Glc*p*NAc-(1→	4.58	3.72	3.58	3.33	3.45	3.71, 3.98
	103.0	57.1	83.1	70.7	78.0	62.9

[a–d] The assignment may be inverse.

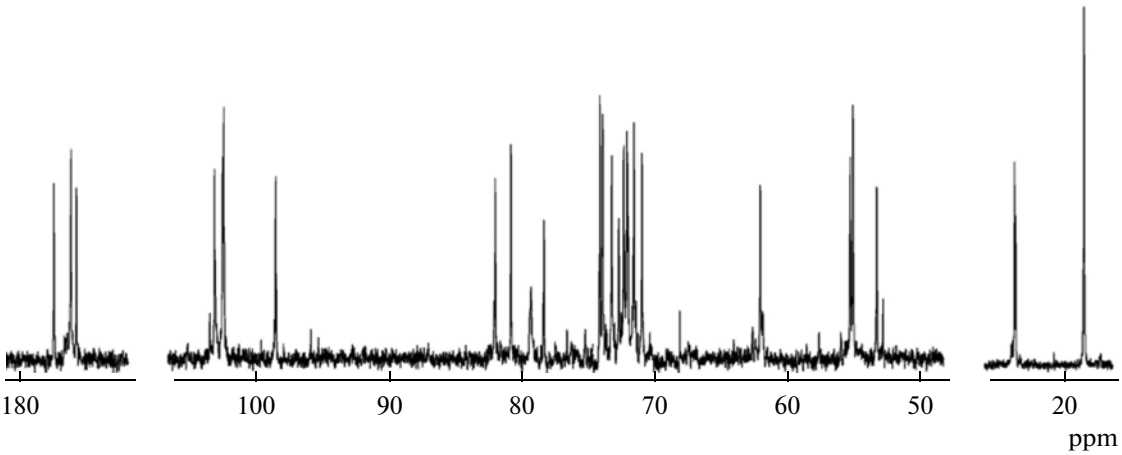

Fig. 2. The ^{13}C-NMR spectrum of the O-polysaccharide from *P. fontium* 27480.

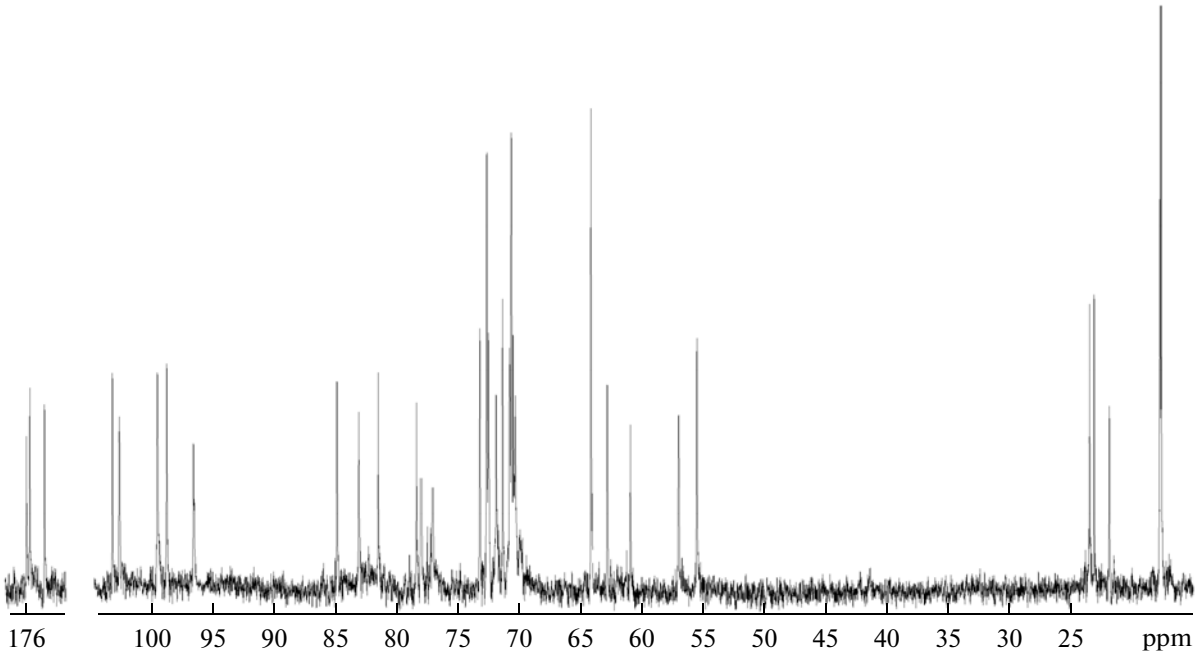

Fig. 3. The ^{13}C-NMR spectrum of the O-polysaccharide from *P. fontium* 97U116.

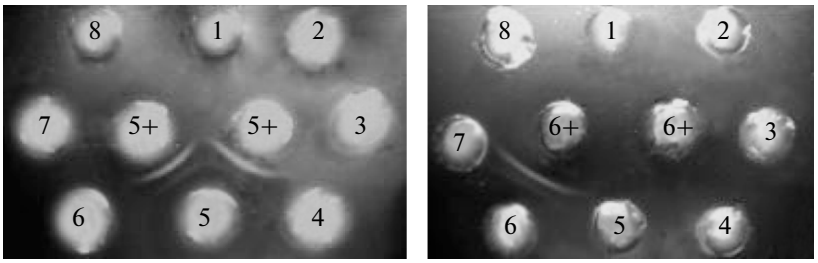

Fig. 4. Double diffusion in agar gel according to the Ouchterlony method for the LPSs from *P. fontium* 97U116 (5) and 27480 (6) with O-antisera to strains 97U116 (5+) and 27480 (6+).

systems. The cross-serological reactions can be applied as an approach to classification of different bacterial species. The Ouchterlony method of double diffusion in agar gel revealed that the LPSs of *P. fontium* 97U116 and 27480 interacted only with the homologous serum (Fig. 4) that indicated the absence of common antigenic determinants complementary to antibodies of another strain; therefore, the studied strains belonged to different serogroups.

Thus, LPSs of two *P. fontium* strains, 97U116 and 27480, were isolated and characterized; they were similar to those of other representatives of *Enterobacteriaceae* in their fatty acid composition. The O-PSs of *P. fontium* 97U116 and 27480 consisted of linear penta- and tetrasaccharide repeating units. The LPSs of the studied strains exhibited serological activity and belonged to different serogroups. The LPSs of *P. fontium* 97U116 and 27480 were less toxic than those of *E. coli* 055:B5 but more pyrogenic than the Pyrogenal preparation.

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