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EXPERIMENTAL ARTICLES

Chemical Characteristics and Endotoxic Activity of the Lipopolysaccharide of *Rahnella aquatilis* 2-95

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Abstract—The lipopolysaccharide (LPS) from a new *Enterobacteriaceae* species, *Rahnella aquatilis* 2-95, was isolated and investigated. The structural components of the LPS molecule, namely, lipid A, core oligosaccharide, and O-specific polysaccharide, were obtained by mild acid hydrolysis. In lipid A, 3-oxytetradecanoic and tetradecanoic acids were found to be the predominant fatty acids. The major monosaccharides of the core oligosaccharide were galactose, arabinose, fucose, rhamnose, and an unidentified component. The O-specific polysaccharide was found to be assembled of a repeated trisaccharide unit of the following structure:

$$\begin{array}{l}\alpha\text{-D-Gal}p\text{-}(1\longrightarrow 2)\text{—}\\ \qquad\longrightarrow 3)\text{-}\alpha\text{-D-Fuc}p\text{-}(1\longrightarrow 3)\text{-}\beta\text{-D-Gal}f\text{-}(1\longrightarrow\end{array}$$

The *R. aquatilis* 2-95 LPS is less toxic and more pyrogenic than the LPS from the *R. aquatilis* 1-95 strain studied earlier. Both acyl and phosphate groups are essential for toxic and pyrogenic activity of *R. aquatilis* 2-95 LPS.

Key words: lipopolysaccharide, *Rahnella aquatilis*, structure, biological activity.

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Populations of microorganisms of the genus *Rahnella* are characterized by significant heterogeneity in the range of circulation and in a number of biological properties. Members of this genus have common phenotypic characteristics but differ in other biological properties, for example, genotypic. For this reason, phenotypically similar *Rahnella* strains are usually divided into genomospecies [1, 2]. This particular term evolved in rahnellae studies and is now being used to designate significant genotypic differences insufficient to discriminate between individual species. According to Pokhil [3, 4], rahnellae also differ in pathogenicity and include pathogenic, slightly pathogenic, and nonpathogenic strains. An important question is how to differentiate between these groups of microorganisms. One of the approaches widely used in laboratory (etiological) diagnoses of infectious diseases caused by enterobacteria is based on serological classification. The main criterion used to divide the strains into serological groups is light differences in the structure of O-specific polysaccharide chains (OPS) of the lipopolysaccharides (LPS). Kaufmann and White were the first to show that the *Salmonella* genus specificity is determined by specific substituent groups in the structure of the OPS, which serves as a basis for differentiating the strains into serological groups. With the progress in carbohydrate chemistry, OPS structures of a number of gram-negative bacteria (for example, *Escherichia coli*, *Citrobacter, Proteus*, *Pseudomonas aeruginosa*, *P. syringae*, etc.) were described and serological classification schemes were developed for them. Until lately, there had been no classification scheme based on O-antigen characteristics for *R. aquatilis*, since none of their OPS have ever been isolated and characterized. *R. aquatilis* is widely spread in nature: they occur frequently in water and soil microbial cenoses, are able to propagate in the gastrointestinal tract of various animals, and cause a wide spectrum of diseases in humans. Thus, we have set up an investigation on the isolation and chemical identification of the *R. aquatilis* LPS. The first structure described in 2004 was an OPS from *R. aquatilis* 1-95 obtained from fresh water [5].

The goal of the present work was to isolate and characterize the LPS from another strain, *R. aquatilis* 2-95, and to determine its OPS structure and certain types of biological activity of the LPS.

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MATERIALS AND METHODS

The subject of the study was a *Rahnella aquatilis* 2-95 culture, isolated from the feces of a clinically healthy individual. It was kindly provided to us by Dr. S. Pokhil (Mechnikov Institute of Microbiology and Immunology, Academy of Medical Sciences of Ukraine).

The culture of *R. aquatilis* was grown on nutrient agar at 28°C for 24 h. Cells were precipitated by centrifugation at 5000 rpm for 30 min and then dried with acetone and ether. Lipopolysaccharides were isolated by the standard water–phenol extraction procedure at 65–68°C [6] followed by removal of nucleic acids by precipitation with trichloroacetic acid and ultracentrifugation at 104000 *g* for 4 h. LPS preparations free of nucleic acids were lyophilized.

Fractions of the OPS and core oligosaccharide were obtained by degradation of the LPS in 3% acetic acid (100°C, 2 h) with subsequent separation of lipid A by ultracentrifugation (25000 *g*, 40 min) and gel filtration of the supernatant on a Sephadex G-50 column (70 × 3 cm) in 0.025 M pyridine acetate buffer solution, pH 4.5.

Nucleic acids were determined according to Spirin [7]; carbohydrates, using phenol and sulfuric acid [8]; protein, by the Lowry method [9]; heptoses, with cysteine and sulfuric acid [10]; and 2-keto-3-deoxyoktanoic acid (KDO), with thiobarbituric acid [11].

Amino acids and amino sugars were quantified after hydrolysis in 6 N HCl for 20 h at 100°C on a KLA-5 amino acids sequence analyzer (Hitachi).

Neutral monosaccharides were identified after hydrolysis in 2 N HCl (105°C, 5 h), or in 2 M CF_3CO_2H (120°C, 2 h). Alditol acetates were analyzed on an Agilent 6890N/5973 inert chromato–mass spectrometry system, DB-225 mS 30 m × 0.25 mm, 0.25 μm column, in 1 a ml/min helium flow. The temperatures of the evaporator, interface, and thermostat were 250, 280, and 220°C, respectively (isothermal regime). The samples were injected into the carrier flow 1 : 100. An Ultra 2 capillary column was used on a Hewlett-Packard 5880 instrument was also used, with a temperature gradient from 180°C (1 min) to 290°C at the rate of 10°C/min [12,13]. Identification of monosaccharides was carried out by comparing alditol acetate retention times of the samples and standards, and using the ChemStation database.

The absolute configurations were determined by GLC of acetylated glycosides with (S)-2-octanol using a Hewlett-Packard 5880 instrument at 230°C (20 min) [14].

Methylation of the polysaccharide was carried out by CH_3I in dimethyl sulfoxide in the presence of methylsulfonyl methanide [15]. The methylated polysaccharide was hydrolyzed in 2 M CF_3CO_2H (100°C, 2 h). Partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett-Packard 5880 instrument equipped with an HP-5ms column using a temperature gradient from 150°C (3 min) to 320°C at 5°C/min.

The NMR spectra of ^1H and ^{13}C were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 27°C. The chemical shifts were recorded with sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) as an internal standard and acetone (δ_C 31.45) as an external standard. Mixing times of 200 and 150 ms were used in total correlation spectroscopy (TOCSY) and Overhauser nuclear effect (NOESY) experiments, respectively.

Lipopolysaccharides were hydrolyzed in acetyl chloride (1.5% methanol solution, 100°C, 4 h), and methyl ethers of fatty acids were analyzed with a computerized Agilent 6890N/5973 inert system (United States).

Dephosphorilation of the LPS was carried out in 40% HF in water (1 ml, 4°C, 24 h). After addition of some water, Dowex 1X4 resin in CO_3^- form was used to bring the solution to neutral pH. The resin was separated by filtration through a glass filter, and the filtrate was frozen and freeze dried [16].

To carry out N,O-deacylation of LPS, it was treated with absolute hydrazine in the presence of hydrazine sulfate (100°C, 40 h). The solution was neutralized with 3M HCl. Fatty acids hydrazides were extracted with $CHCl_3$. After removal of salts on a Sephadex G-50 gel-filtration column, deacylated LPS samples were freeze dried [17].

Antisera to the heated (100°C, 2.5 h) *R. aquatilis* culture were obtained by four intravenous immunizations of rabbits (2.5 kg) with increasing doses of bacterial suspensions (2×10^6 to 5×10^7 cells/ml) with seven-day intervals. The animals were exsanguinated on the seventh day after the last injection. Double diffusion in agar was carried out as described by Ouchterlony [18] in 1% agarose gel in physiological saline with 2% polyethylene glycol 6000. Immunophoresis in 1% agarose gel was performed in 0.05 M veronal–medinal buffer solution, pH 8.6, at 80–95 V (12 mA). After electrophoresis, the native antiserum was introduced into each pit and the gel was left overnight at 37°C for precipitation to develop [19].

White outbred mice sensitized with galactosamine were used to estimate the LPS toxicity (LD_{50}).

Pyrogenicity of the LPS was studied in rabbits by intravenous injection of the minimal pyrogenic dose of LPS followed by thermometry of the animals over 3 h.

RESULTS AND DISCUSSION

The yield of *R. aquatilis* 2-95 LPS isolated by the water–phenol method was as high as 17.5%, while it was only 5.0% for other gram-negative bacteria and 7.7% for the *R. aquatilis* 1-95 LPS studied by us earlier. The specific nature of the extraction method has led to a relatively high nucleic acid content in the extract (up to 30%). On the other hand, it is probably characteristic of *R. aquatilis* LPS to contain a lot of nucleic acids.

Table 1. Monosaccharide composition of the *R. aquatilis* 2-95 LPS structural components

Sample	Monosaccharides, % of the total peak area										Monosaccharides, % of the sample dry weight		
	Rham	Fuc	Rib	Arab	Xyl	Man	Gal	Glu	X_1	X_2	KDO	Heptoses	Glucosamine
LPS	–	16.6	4.2	5.4	5.8	1.9	37.7	15.6	–	–	0.12	7.6	0.42
OPS	–	28.8	–	–	–	–	71.2	–	–	–	–	–	–
Core oligosaccharide II	12.3	1.7	–	–	–	–	37.5	20.9	25.8	25.8	–	25.5	*
Core oligosaccharide III	–	20.8	–	3.7	–	–	69.9	5.6	–	–	0.75	–	*

Note: "–" stands for "not present". * stands for "wasn't studied". X_1, X_2 are the monosaccharides eluted after glucose.

Nucleic acids were removed by ultracentrifugation and precipitation of insoluble complexes with trichloroacetic acid. This method of LPS purification is rather effective, but leads to a significant loss in the LPS yield dew to partial coprecipitation of LPS and nucleic acids. The purified LPS preparation was shown to contain 55.2% of carbohydrates, 4.0% of nucleic acids, and trace amounts of protein. According to an analysis of the composition of monosaccharides (Table 1), galactose (37.7%), fucose (16.6%), and glucose (15.6%) were the predominant monosaccharides in the *R. aquatilis* 2-95 LPS. The content of other monosaccharides, such as xylose, arabinose, ribose, and mannose, was 5.8, 5.4, 4.2, and 1.9%, respectively. Glucosamine content was low (0.42%). Heptose was shown to be another characteristic component of *R. aquatilis* 2-95 LPS (7.6%). The undegraded LPS preparation also contained 0.12% of KDO.

Thus, the *R. aquatilis* 2-95 LPS contained all typical components of these biopolymers.

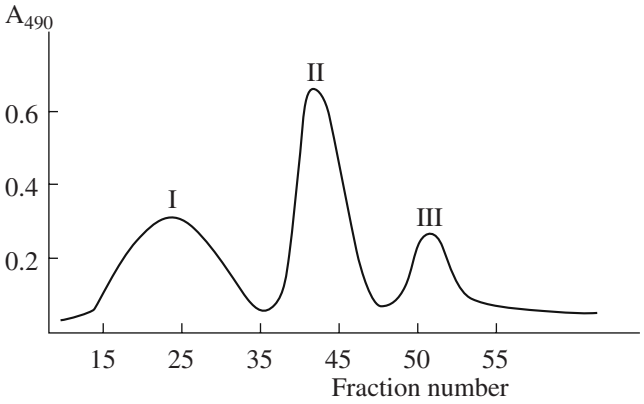


Fig. 1. Degraded *R. aquatilis* 2-95 LPS Sephadex G-50 elution profile: I, O-specifis oligosaccharide fraction; II and III, core oligasaccharide fractions.

The evaluation of the amino acid content, along with the Lowry assay, revealed negligible amounts of protein in *R. aquatilis* 2-95 LPS.

Soft acid hydrolysis makes it possible to isolate individual components of the LPS macromolecule by breaking the ketoside bond between KDO, the core oligosaccharide component, and the C6-OH group in glucosamine II of lipid A. The precipitate of lipid A was separated by ultracentrifugation. Gel chromatography was used to separate water-soluble products of the hydrolysis of the LPS. The presence of both a high molecular weight OPS fraction (fraction I) and low molecular weight fractions of the core oligosaccharide (fractions II and III) proves that the LPS represents a mixture of S- and R-type molecules (Fig. 1).

Lipid A is the most conservative part of the LPS molecule. Thus, variations in the fatty acid composition of lipid A may be an extra taxonomic criterion for species differentiation. According to the results of chromatography–mass spectrometry analysis of the methyl ethers of fatty acids (Table 2), *R. aquatilis* 2-95 LPS contains fatty acids with C12- to C16-long chains. In lipid A of the bacteria under study, 3-oxytetradecanoic (52.4%), tetradecanoic (28.7%), dodecanoic (14.8%), and hexadecanoic (4.1%) acids were identified. The presence of only one hydroxylated fatty acid, 3-oxytetradecanoic acid, is typical of enterobacteria; it acylates both amino and hydroxy groups of glucosamine residues. Thus, our experimental data on the composition of fatty acids prove the correctness of considering *R. aquatilis* 2-95 a representative of enterobacteria.

In the LPS molecule, lipid A is known to act as the endotoxic center and it is lipid A which determines the lethal toxicity and pyrogenicity of the LPS. In experiments with mice sensitized with D-galactosamine (Table 3), the *R. aquatilis* 2-95 LPS was shown to be less toxic (6 μg/mouse) compared to the *E. coli* O55:B5 (Sigma) LPS used as a positive control (0.14 μg/mouse). In an analysis of the pyrogenicity, the temperature shift in the case of *R. aquatilis* 2-95 LPS was +0.81, +1.0, and +0.81 degrees in 1, 2, and 3 h after injection, respectively. For Pyrogenalum, a commercial

pharmaceutical based on the *Shigella typhi* LPS, the shift was +0.59, +0.69, and +0.71, respectively. Thus, the LPS under study is more pyrogenic than the control. *Rahnella aquatilis* 2-95 LPS was also shown to be less toxic and more pyrogenic than *R. aquatilis* 1-95 LPS studied by us earlier. The toxicity of *R. aquatilis* 1-95 LPS was 5.0 μg/mouse, the temperature shift in 1, 2, and 3 h after injection was +0.70, +0.54, and +0.50 degrees, respectively.

Lipopolysaccharides of gram-negative bacteria possess a wide spectrum of biological activity: such as radioprotector, pyrogenic, immunomodulating, antitumor, etc. [20, 21]. Therefore, their medicinal applications are presently under consideration. A major obstacle to this is high LPS toxicity. Investigators are thus forced to search for new and less toxic polymers, or to develop modified, a less toxic LPS with the same therapeutic effect. Since no information was found in the literature concerning the effect of lipid A individual components on its toxicity and pyrogenicity, except for our study of *R. aquatilis* 1-95 LPS [5], we have obtained dephosphorylated and N,O-deacylated derivatives of the LPS molecules. These modified LPS are characterized by complete loss of toxicity and pyrogenicity (Table 3). This means that both phosphate and acyl groups are obligatory for the toxic and pyrogenic effects of the LPS. A study of the fatty acid composition presently cannot answer the question of which fatty acids are essential for the biological activity under study.

Compared to lipid A, the core oligosaccharide is a more variable part of the LPS molecule and its structure had not been thoroughly studied until the recent development of new research methods. In the present-day view, all LPS regardless of their bacterial origin contain at least 2-keto-3-desoxyoctanoic acid (KDO) residue or its derivative with an open carboxyl group through which binding to the polysaccharide part of the lipid A molecule occurs. Bacteria with defective KDO are not viable. Thus, KDO and the LPS as a whole are essential for the structural integrity and functioning of bacterial cells. According to the latest research [22], in most core oligosaccharides, though not in all of them, L- or D-glycero-D-mannoheptose is present. We have shown that fraction II (Table 1) of the *R. aquatilis* 2-95 core oligosaccharide contains galactose (37.5%), an unidentified component (25.8%), glucose (20.9%), and rhamnose (12.3%); fraction III contains galactose (69.9%), fucose (20.8%), glucose (5.6%), and arabinose (3.7%). Earlier, galactose was found in the structure of *Salmonella enterica, S. sonnei, S. flexneri* strains 6, R4, and 4b, *E. coli* R1 and R2, *Citrobacter* O27 and PCM 1487, O23, *Erwinia carotovora* FERM P-7576, and *Yersinia enterocolitica* core oligosaccharide; arabinose was found in core oligosaccharides of *Proteus mirabilis* R45/1959, *Pseudomonas fluorescens*, and *Ralstonia solanacearum* [20]. Heptoses and KDO were found in oligosaccharide fractions II and III, respectively. Low values of KDO content in *R. aquatilis* 2-95 core oligosaccharide are probably due to the presence of a KDO residue with a substituent at the C5 atom, which leads to low response in the reaction with thiobarbituric acid. The comparative analysis of core oligosaccharides derived from *R. aquatilis* strains 2-95 and 1-95 studied earlier [5] indicates qualitative similarity but differences in quantities.

Table 2. Fatty acid composition of *R. aquatilis* 2-95 LPS (% of the total peak area)

Sample	Fatty acids			
	$C_{12:0}$	$C_{14:0}$	3OH-$C_{14:0}$	$C_{16:0}$
LPS	14.8	28.7	52.4	4.1

Table 3. Toxicity and pyrogenicity of the native and modified *R. aquatilis* 2-95 LPS

Sample, strain		LD_{50}, μg/mouse	Pyrogenicity			
			LPS dose, μg/ml	Temperature shift (°C)		
				1 h	2 h	3 h
R. aquatilis LPS	native	6.0	7.5×10^{-3}	+0.81	+1.0	+0.81
	dephosphorylated	6.0	7.5×10^{-3}	–0.07	–0.03	–0.06
	N, O-deacetylated	6.0	7.5×10^{-3}	–0.45	–0.39	–0.60
E. coli O55:B5 LPS		0.14	–	–	–	–
Pyrogenalum		–	7.5×10^{-3}	+0.59	+0.69	+0.71

Notes: In toxicity measurements, *R. aquatilis* 2-95 LPS was injected into the caudal vein, *E. coli* LPS was injected intraperitoneally. "–" stands for "wasn't studied".

The most variable part of the LPS molecule is the O-specific polysaccharide (OPS), which determines the antigen specificity of the bacterial cell. The vast variability in structure of OPS underlies the classification of bacterial strains into different serological groups. According to the results of chromato–mass spectrometry analysis of the alditol acetates obtained by complete acid hydrolysis of the polysaccharide, OPS contains fucose and galactose in the ratio of 1 : 2.

Determination of the absolute configuration of acylated (S)-2-octyl glycosides indicated that both monosaccharides have the D configuration.

Methylation analysis, including GLC of partially methylated alditol acetates, revealed derivatives of 4-O-methylfucopyranose, 2,3,4,6-tetra-O-methylgalactopyranose, and 2,5,6-tri-O-methylgalactofuranose. Therefore, the OPS is a branched one, with a terminal galactopyranose residue in the side chain, 2,3-disubstituted

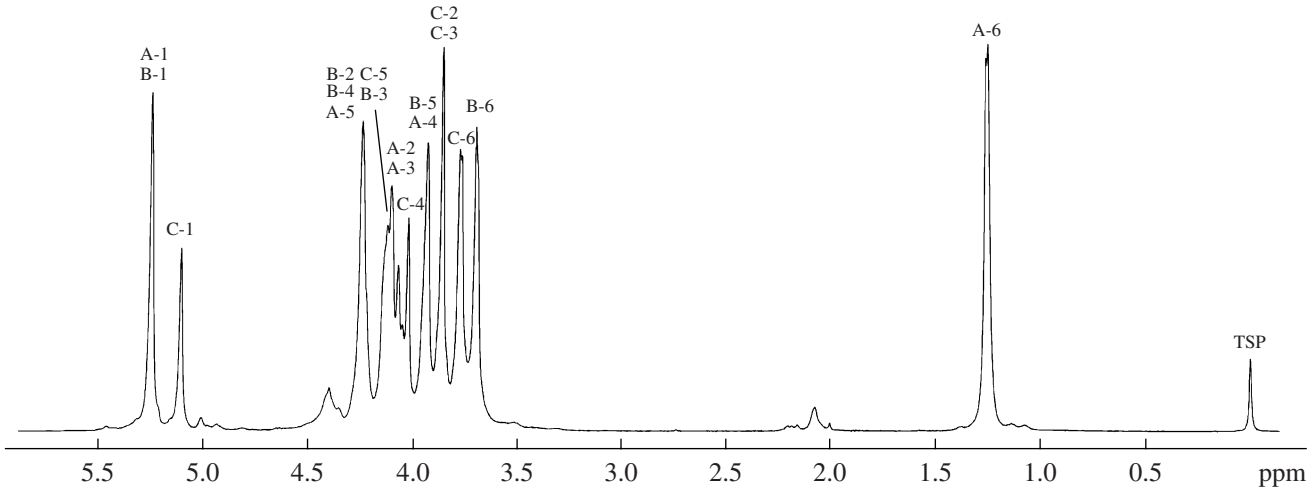


Fig. 2. *R. aquatilis* 2-95 OPS ^{1}H NMR spectrum.

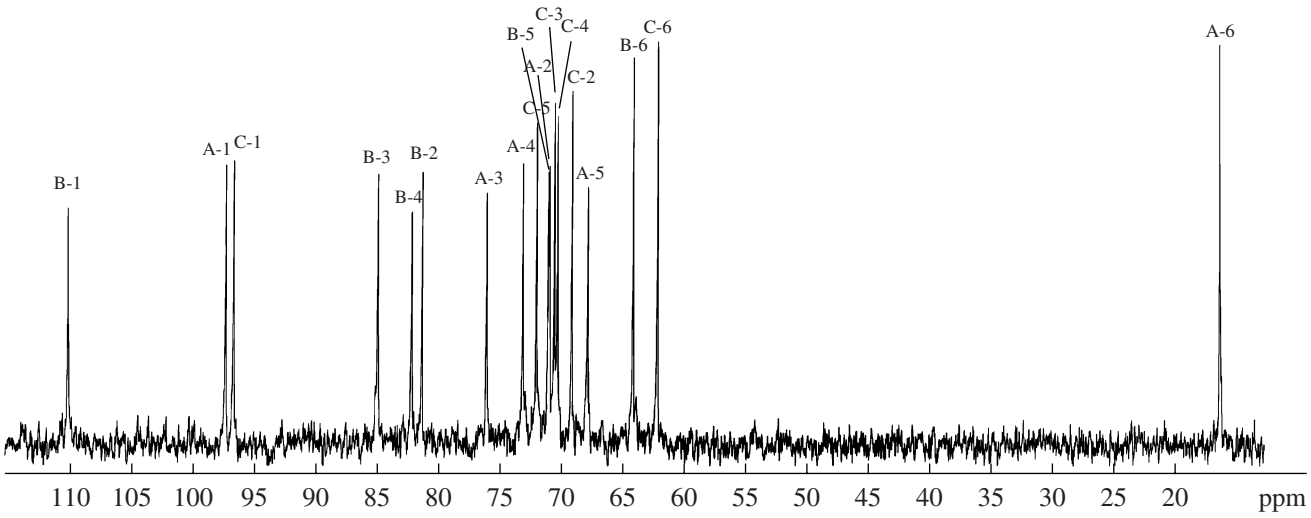


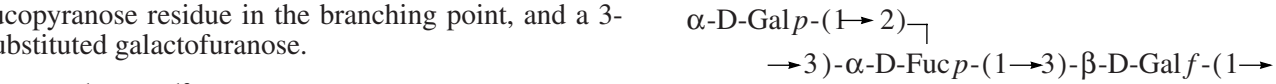
Fig. 3. *R. aquatilis* 2-95 OPS ^{13}C NMR spectrum.

fucopyranose residue in the branching point, and a 3-substituted galactofuranose.

Both ^{1}H and ^{13}C NMR spectra (Figs. 2 and 3) were identical to those of *R. aquatilis* 1-95 OPS, the structure of which had been established earlier [5].

Summing up the results of the chemical analysis (identification of the monosaccharide composition and methylation) and NMR spectrometry, the *R. aquatilis* 2-95 OPS is a regular polysaccharide; the repeating unit is a branched trisaccharide consisting of two D-Gal and one D-Fuc residues with a galactopyranose residue in the side chain. The 2-, 3-disubstituted D-fucopyranose residue is located in the branching point. D-galactofuranose residue is 3-substituted.

Therefore, the structure of the *R. aquatilis* 2-95 O-specific polysaccharide is as follows:



The lipopolysaccharide is the major thermostable antigen of the bacterial cell. The serological specificity of the LPS is determined by the OPS structure. To evaluate the correlation between OPS structural specificity and LPS serological properties of three strains (*R. aquatilis* strains 1-95 and 2-95 with similar OPS structure, and the type strain 33071, the OPS structure of which is not yet established), high-titre (1 : 12800 and 1 : 25600 in ring precipitation and agglutination reactions, respectively) antisera were obtained. Double immunodiffusion in agar and immunoelectrophoresis (for strain 2-95) showed that LPS of the strains under study exhibited antigen activity in a homological system. Serological cross-reactivity based on the phylogenetic relations is known to have been used as one of the

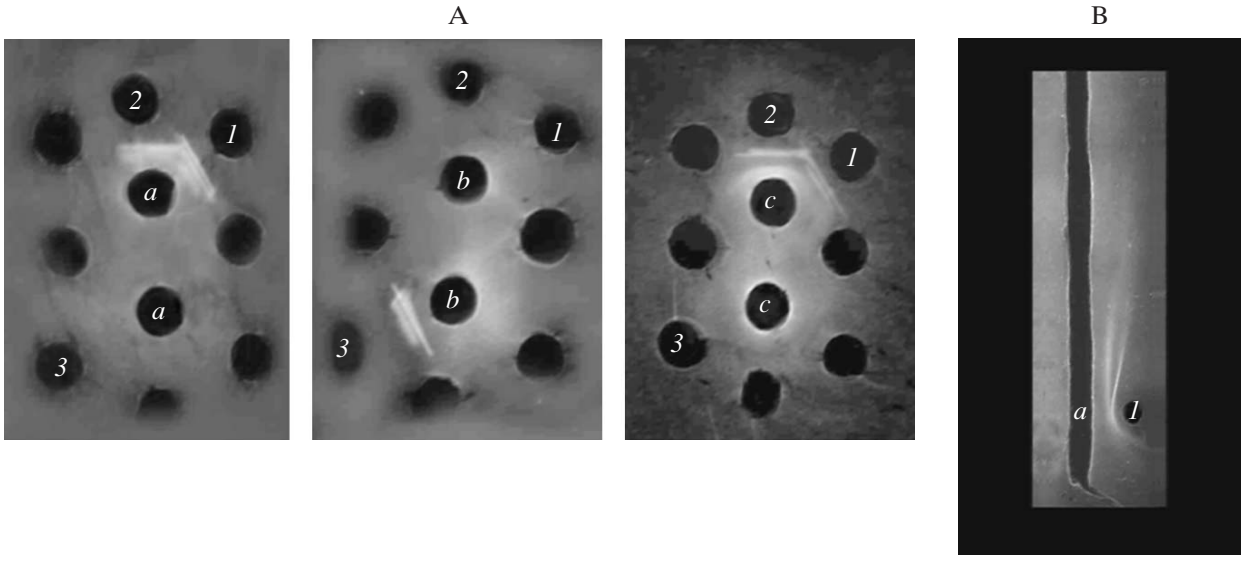


Fig. 4. (A) Double immunodiffusion in agar of *R. aquatilis* strains 2-95 (1), 1-95 (2), and 33071 (3) LPS; (B) immunoelectrophoresis with antisera against strains (*a*) 2-95, (*b*) 33071, and (*c*) 1-95; (B) *R. aquatilis* 2-95 immunoelectrophoresis with homological antiserum.

complementary methods in species classification along with the traditional taxonomic methods. In double immunodiffusion serological cross-reactions *R. aquatilis* 2-95 LPS that reacted with the antiserum against *R. aquatilis* 1-95 and visa versa, indicating that there are common antigens in the structure of these LPS OPS. Both *R. aquatilis* 1-95 and 2-95 LPS did not react with the antiserum against type strain 33071, and the latter did not react with either the *R. aquatilis* 1-95 or the 2-95 LPS antisera. Thus, the type strain antiserum probably does not contain antibodies against *R. aquatilis* 2-95 and 1-95 LPS (Fig. 4).

Two *R. aquatilis* strains have been isolated from different sources: *R. aquatilis* 2-95, from the feces of a clinically healthy individual, and 1-95, from water of the Uda River (Kharkov). According to a comparative analysis of the LPS characteristics of these strains, they are very similar. Both LPS contain molecules in both S- and R-forms, and they are characterized by similar fatty acid composition and OPS structure. The LPS of both strains exhibit serological cross-reactivity, so they possess common antigen determinants and should be classified in the same serological group. The type strain *R. aquatilis* 33071 did not exhibit serological affinity to strains 2-95 and 1-95 and probably belongs to another serological group. *Rahnella aquatilis* 2-95 LPS is less toxic and more pyrogenic than *R. aquatilis* 1-95 LPS. Since the toxicity and pyrogenicity are determined by the structure of lipid A, our further investigation will focus on the specificities of the structure of lipid A.

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