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EXPERIMENTAL ARTICLES

Structure and Properties of the Lipopolysaccharide of *Pseudomonas fluorescens* IMV 2366 (Biovar III)

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Abstract—The lipopolysaccharide (LPS) preparation isolated from the bacterial mass of *Pseudomonas fluorescens* IMV 2366 (biovar III) by Westphal's method and purified by repeated ultracentrifugation contained S- and R-forms of molecules. The structural components of the LPS molecule—lipid A, core oligosaccharide, and O-specific polysaccharide—were obtained in the individual state and characterized. The main components of the lipid A hydrophobic moiety were 3-hydoxydecanoic, 2-hydroxydodecanoic, 3-hydroxydodecanoic, dodecanoic, and hexadecanoic fatty acids. Glucosamine, phosphoethanolamine, and phosphorus were identified as the components of the lipid A hydrophilic moiety. Rhamnose, glucose, galactose, glucosamine, galactosamine, alanine, phosphoethanolamine, phosphorus, and 2-keto-3-deoxyoctulosonic acid (KDO), as well as 2-amino-2,6-dideoxygalactose (FucN) and 3-amino-3,6-dideoxyglucose (Qui3N), were revealed in the composition of the core oligosaccharide fractions. O-specific polysaccharide chains were composed of repeating trisaccharide units consisting of residues of L-rhamnose (L-Rha), 2-acetamido-2,6-dideoxy-D-galactose (D-FucNAc), and 3-acylamido-3,6-dideoxy-D-glucose (D-Qui3NAcyl), where Acyl = 3-hydroxy-2,3-dimethyl-5-hydroxyprolyl. Neither double immunodiffusion in agar not the immunoenzymatic assay revealed serological relations between the strain studied and the *P. fluorescens* strains studied earlier.

Key words: *Pseudomonas fluorescens*, lipopolysaccharide, lipid A, core oligosaccharide, O-specific polysaccharide.

The bacteria of the species *Pseudomonas fluorescens* are saprophytic gram-negative microorganisms heterogeneous with respect to their phenotypic and genotypic characteristics. According to the international classification, the bacteria of this taxon are subdivided into five biovars with an inconclusively established taxonomic rank [1]. The strains assigned to biovar III (biotype C according to Stanier *et al.* [2]), like other representatives of this species, have been isolated from different sources (waters, soils, food products, plant rhizospheres). The DNA homology level of *P. fluorescens* biovar III and strains representing other biovars of the species does not exceed 60% [3]. The level of similarity according to the data on the structure of the lipopolysaccharides (LPS) isolated from the bacteria of individual strains also does not exceed 60% [4]. LPS are one of the main components of the cell envelope of gram-negative bacteria; their biosynthesis is determined by genes of conservative nature. Therefore, data on LPS structure and biochemical properties may be used as a chemotaxonomic criterion. An LPS molecule includes a hydrophilic carbohydrate domain, in which the O-specific polysaccharide and the core oligosaccharide are distinguished. The latter is covalently bonded to the LPS hydrophobic portion, lipid A, which serves as a link between the carbohydrate domain and the outer cell membrane. LPS molecules are brought into direct contact with the environment due to their localization in the outer monolayer of the bacterial cell membrane. These biopolymers form complexes with protein macromolecules and are excreted by a microbial cell in its lifetime and after cell death into the environment [5]. To date, numerous data have been accumulated on the structure and biological properties of the LPS isolated from different species of gram-negative bacteria. However, the LPS of *P. fluorescens* remain relatively little studied. Used for many species of gram-negative bacteria, the classification based on the serological properties of the LPS O-specific polysaccharide chains has not been developed for *P. fluorescens* bacteria. Earlier [4, 6–12], we studied the LPS of the strains representing different biovars of *P. fluorescens*, including the type strain IMV 4125 = ATCC 13525, representing biovar I, and strain IMV 2125, assigned to biovar III. The macromolecular organization and specific features of the structure of individual structural portions of the LPS macromolecule were investigated. Similarity was revealed between LPS from different *P. fluorescens* strains with respect to the lipid A fatty acid composition and the lipid A hydro-

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philic portion components. As for the composition of the core and the structure of the O-specific polysaccharide chain, the LPS studied earlier showed a certain level of heterogeneity.

In this work, the results of our studies of the LPS of *P. fluorescens* IMV 2366 (biovar III) are presented. The aims of the work were to isolate the LPS and its structural parts and to study the specific features of the composition and structure of lipid A, core oligosaccharide, and O-specific polysaccharide chains; to characterize the serological properties of the LPS; and to estimate the degree of similarity between the LPS of the strain studied and the LPS of *P. fluorescens* IMV 2125 (biovar III) (studied in part earlier) for further use of the findings in the classification of these bacteria.

MATERIALS AND METHODS

Strain *Pseudomonas fluorescens* IMV 2366 (biovar III) was obtained from the collection of microorganisms maintained at the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. The bacteria were grown in nutrient agar (NA) at 28°C for 28 h. The bacterial mass was washed off with saline; the suspension was centrifuged for 30 min at 6000 rpm, washed with saline, and dried with acetone and diethyl ether. The O-sera were prepared by immunizing rabbits with suspensions of microbial cells killed by heating (4×10^9 cells/ml, 100°C, 2.5 h). Serological studies were performed according to the techniques described in [6, 12]. LPS from dry bacterial mass were isolated by phenol–water extraction [13] at 65°C and purified by triple ultracentrifugation at 105000 *g*. The precipitate was dried lyophilically. To isolate the fractions of lipid A, core oligosaccharide, and O-specific polysaccharide, the LPS preparation was subjected to mild acid hydrolysis with 1% acetic acid (1.5 h, 100°C). The water-insoluble lipid A fraction was separated by centrifugation at 6000 rpm. The fractions corresponding to the core oligosaccharide and O-specific polysaccharide were isolated by gel filtration of LPS carbohydrate moiety on a column with Sephadex G-50. The methylation of lipid A fatty acids was carried out in sealed ampoules with 1.5 M HCl in methanol (100°C, 3 h); the methyl ethers of fatty acids were analyzed using a Chrom-5 gas chromatograph (CSSR) with a flame ionization detector on a 1.2-m × 3-mm column with 5% SE-30 on Chromaton N-AW-DMCS, as well as on a 2-m × 3-mm column with 5% DEGS-PS on Supelcoport (100–200 mesh). Helium was used as the carrier gas. Trifluoroacetylation of methyl esters of hydroxy acids was carried out as described in [11]. The derivatives obtained were analyzed on a gas chromatograph under the conditions described above. KDO was determined by the reaction with thiobarbituric acid [14]. The total carbohydrate content in the LPS preparations and the phosphorus content were determined as described in [7]. Neutral sugars in the hydrolysates (2 N HCl, 4 h, 100°C) of the LPS carbohydrate component preparations were analyzed by ion-exchange chromatography on a column with the BTA 2118 anion-exchange resin (Biotronik, Germany) in a step-gradient system of potassium–borate buffers according to the technique described in [10] and by GLC in the form of acetate polyols, as described in [6]. The absolute configuration of monosaccharides was determined after their derivation into acetylated (R)-2-octyl glycosides and GLC as described in [15]. ^1H and ^{13}C NMR spectra were recorded in D_2O at 45°C using a Bruker DRX-500 device. Acetone (δ_n 2.225 ppm, δ_c 31.45 ppm) was used as the internal standard. The spectrophotometric analysis of the LPS preparation was carried out using a DU-8B spectrophotometer (Beckman, USA).

RESULTS AND DISCUSSION

The LPS preparation of *P. fluorescens* IMV 2366 (biovar III) isolated from the dry bacterial mass with hot phenol and purified by ultracentrifugation showed serological activity in reactions of double immunodiffusion in agar and immunoenzymatic analysis with homologous O-serum obtained by immunizing the rabbits with bacterial cells inactivated by heating, but in cross reactions with O-sera against the neotype strain *P. fluorescens* IMV 4125 and the strains representing the other biovars of the species, including strain IMV 2125 (biovar III), the LPS studied was inactive.

Spectrophotometrically, the LPS preparation (1 mg/ml solution) was characterized by an absorption band in the range 200–220 nm; the admixture of nucleic acids and the protein component were below the detection level. The LPS preparation contained 46% carbohydrates when determinations were carried out by a reaction with phenol and sulfuric acid and 4.1% phosphorus. The LPS yield was 3.4% of the bacterial mass dry acetone powder.

The water-insoluble lipid A fraction isolated after degradation of the initial LPS preparation with 1% acetic acid at 100°C for 1.5 h constituted 48% of the mass of the lyophilized LPS preparation.

Using the method of ion-exchange chromatography, we identified glucosamine and phosphoethanolamine as the main components of the hydrophilic portion of the lipid A preparation (Fig. 1). Ethanolamine and the amino acids (asparagine, serine, glycine, alanine, leucine, glucosamine, histidine, lysine) commonly occurring in microbial membrane proteins were present in minor amounts. The quantitative ratio of the main components and the phosphorylation level (Table 1) were the same as in lipid A of the other strains representing biovars I and II of the species *P. fluorescens* and studied by us earlier [8–10, 12].

Based on the data from GLC and chromatography–mass spectrometry, fatty acids (Table 2) were identified in lipid A of the LPS studied. These fatty acids were also revealed in the other *P. fluorescens* representatives, in particular, in lipid A of the type strain IMV 4125

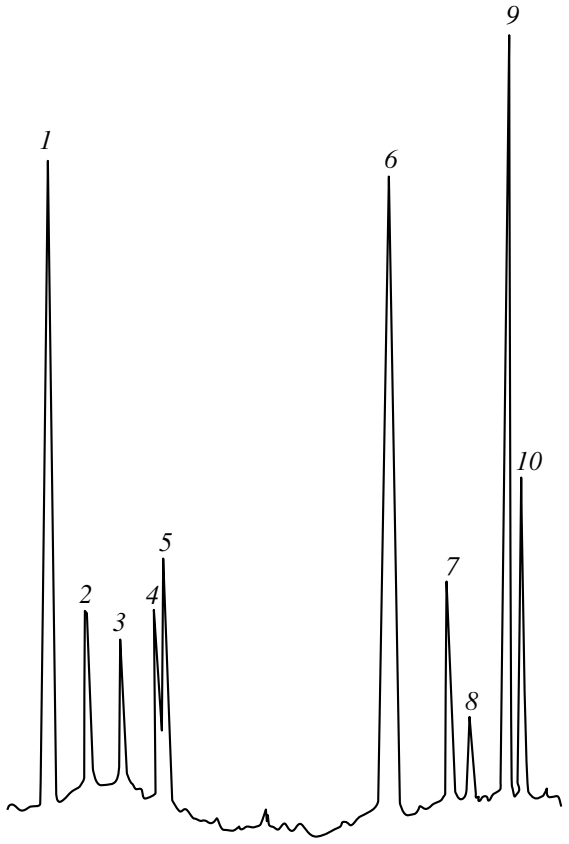

Fig. 1. Chromatogram of the separation of hydrolysate components (4 N HCl, 6 h) of lipid A from the LPS of *P. fluorescens* IMV 2366 (biovar III) on the amino acid analyzer in the system of sodium–citrate buffers: (*1*) phosphoethanolamine; (*2*) asparagine; (*3*) serine; (*4*) glycine; (*5*) alanine; (*6*) glucosamine; (*7*) histidine; (*8*) lysine; (*9*) ammonia; (*10*) ethanolamine.

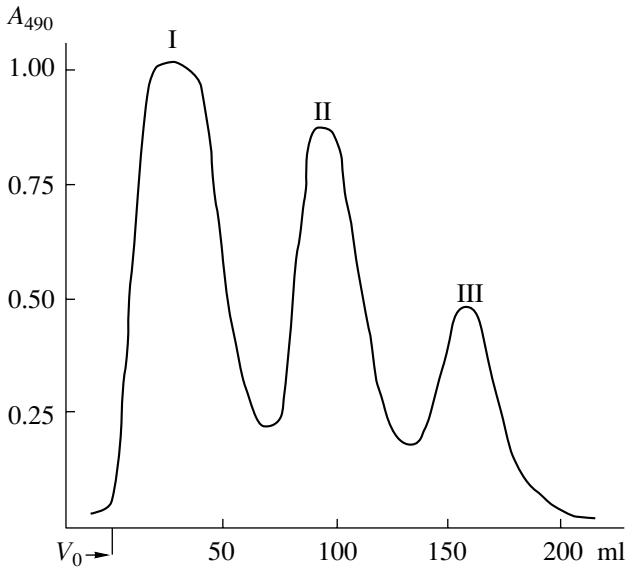

Fig. 2. Elution profile of gel filtration of the carbohydrate portion of *P. fluorescens* IMV 2366 (biovar III) LPS on a column with Sephadex G-50. I, O-specific polysaccharide fraction; II and III, core oligosaccharide fractions; V_0, free volume.

(biovar I) and IMV 2125 (biovar III) [11]. An increased amount of hexadecanoic acid (18.9%) may be considered to be a specific feature of lipid A of the strain studied. But in the composition of predominant fatty acids (3-hydroxydecanoic, 2-hydroxydodecanoic, 3-hydroxydodecanoic, dodecanoic, and hexadecanoic fatty acids), the lipid A of IMV 2366 is virtually identical to lipid A of *P. fluorescens* type strain IMV 4125 and *P. fluorescens* IMV 2125 assigned to biovar III; this, together with the similarity in structure of the hydrophilic portions, may give evidence of similar structural organization of the lipids A of *P. fluorescens* bacteria belonging to different biovars.

As seen from Fig. 2, the elution profile of the LPS carbohydrate component upon gel filtration on a column with Sephadex G-50 indicates that the initial preparation is a mixture of the S- and R-forms of LPS molecules. As was established earlier [16], the same LPS macromolecular organization is characteristic of *P. fluorescens* IMV 2125 belonging to biovar III, as well as for the LPS of biovar I strains [12]. Thus, taking into account the identical conditions for growing bacteria and methods for LPS isolation and purification that allow comparative study of the LPS of different *P. fluorescens* strains to be conducted, we may conclude that the presence of different forms of molecules in a complex LPS preparation is also characteristic of biovar III representatives.

The core oligosaccharide fraction was eluted upon gel filtration on a column with Sephadex G-50 with two peaks (Fig. 2) whose component composition was virtually identical. Their composition revealed the components (Table 3) identified in this portion of the LPS macromolecule of the other *P. fluorescens* strains [7–10, 12]. These are primarily rhamnose and alanine, revealed in the LPS core of all *P. fluorescens* strains studied by us earlier. Certain differences were observed in the component composition of the core of the studied

Table 1. Components of the hydrophilic moiety of lipid A of *P. fluorescens* IMV 2366 (biovar III)

P. fluorescens (biovar III), IMV	Glucosamine		Phosphoethanolamine		Phosphorus	Amino acids
	%	mM	%	mM	%	%
2366	5.10	0.26	1.81	0.12	3.70	2.00

strain relative to the LPS core of strain IMV 2125, also representing biovar III (Table 3). In the former, no fucose was revealed; only trace amounts of rhamnose and heptose were detected; and glucosamine was found (only trace amounts of it occurred in the core of the comparison strain IMV 2125). The alanine content, higher than in the core of strain IMV 2125, may be explained by a difference in the content (a greater amount) of LPS-conjugated membrane proteins. In addition to the components revealed in the LPS core oligosaccharides of the strains studied earlier, 2-amino-2,6-dideoxygalactose (FucN) and 3-amino-3,6-dideoxyglucose (Qui3N) were identified in the core studied using ion-exchange chromatography. It should be noted that these sugars were also revealed in the core oligosaccharide of *P. fluorescens* ATCC 49271 characterized at the structural level [17]. However, the core oligosaccharide of this strain differs from the other LPS core oligosaccharides of *P. fluorescens* strains by the absence of rhamnose. Thus, the data obtained by us, along with the literature data, give evidence of the heterogeneity of this portion of the LPS macromolecule in the representatives of *P. fluorescens* bacteria.

In the acid hydrolysate of the O-specific polysaccharide fraction isolated by gel filtration of the carbohydrate portion of the LPS macromolecule on a column with Sephadex, FucN and Qui3N were identified on the amino acid analyzer in the system of sodium–citrate buffers by comparing the retention times with the corresponding standards. Alanine was revealed in minor amounts. When the components of this hydrolysate were analyzed in the form of borate complexes by high-performance liquid chromatography (HPLC), as well as by GLC in the form of polyol acetates, rhamnose was also revealed in its composition. It followed from these data that the O-specific polysaccharide consisted of rhamnose, 2-amino-2,6-dideoxygalactose, and 3-amino-3,6-dideoxyglucose residues. Apparently, the minor components were revealed due to the presence of the core oligosaccharide components in the polysaccharide, which was determined by the LPS macromolecular organization and the conditions of mild acid hydrolysis with acetic acid cleaving the acid-labile bond between lipid A and the macromolecular carbohydrate portion [7].

In the polysaccharide ^{13}C NMR spectrum (Fig. 3, Table 4), the resonance region of anomeric carbon atoms had three signals: 96.7, 103.6, and 105.8 ppm; two signals at 49.4 and 57.9 ppm corresponding to CHNH groups; the CH_2NH signal at 46.2 ppm; six CH_3–C signals at 16.9, 18.1, 18.4, 19.4, 23.7, and 23.9 ppm; three signals at 175.3, 176.0, and 180.3 ppm corresponding to CO groups; and the signals of 12 other carbon atoms in the 68.7–81.6 ppm region. The ^1H NMR spectrum contained the signals of three anomeric protons at 4.81, 4.82 (undivided), and 5.62 ppm; five CH_3–C groups at 1.23, 1.27, 1.30, 1.36, and 1.47 ppm; one C–CH_2–C group at 2.42 and 2.71 ppm; and one *N*-acetyl group at 2.05 ppm (Fig. 4, Table 5). It followed from these data that the polysaccharide consists of repeating units made up of three residues of 6-deoxy sugars and includes an unusual substitute of noncarbohydrate nature. The comparison between the chemical shifts in the ^1H and ^{13}C NMR spectra of the polysaccharide studied and the data of the NMR spectra of the *P. fluorescens* 361 O-specific polysaccharide [15] showed that the noncarbohydrate component was the *N*-bonded 3-hydroxy-2,3-dimethyl-5-hydroxyprolyl group (Table 5).

After hydrolysis of the polysaccharide and conversion of the monosaccharides to polyol acetates, with subsequent analysis of the derivatives obtained with the HPLC–mass spectrometry method, rhamnose, 2-acet-

Table 2. Fatty acid composition of lipid A of *P. fluorescens* (biovar III) (% of total fatty acids)

Peak no., fatty acid	*P. fluorescens* IMV	
	2366	2125 [11]
1, 3–OH–$C_{10:0}$	16.40	10.20
2, $C_{12:0}$	14.20	23.60
3, 2–OH–$C_{12:0}$	20.00	20.80
4, 3–OH–$C_{12:0}$	24.00	23.40
5, $C_{16:1}$	2.70	6.60
6, $C_{16:0}$	18.90	11.10
7, $C_{18:1}$	3.00	4.20
8, $C_{18:0}$	0.80	0.10

Table 3. Composition of the core oligosaccharide of *P. fluorescens* (biovar III)

Component (% of total)	*P. fluorescens* IMV	
	2366	2125 [11]
Rhamnose	64.94	52.0
Galactose	3.11	1.2
Glucose	31.95	3.6
Heptose	traces	3.0
Mannose	traces	1.2
Fucose	–	39.0
(% of dry preparation weight)		
Alanine	8.5	1.8
Glucosamine	7.5	traces
Galactosamine	4.9	6.7
Phosphoethanolamine	0.6	3.6
2-Keto-3-deoxyoctulosonic acid	5.9	4.6
2-Amino-2,6-dideoxygalactose	6.1	–
3-Amino-3,6-dideoxyglucose	3.1	–
Phosphorus	4.8	3.7

Note: "–" means "not detected."

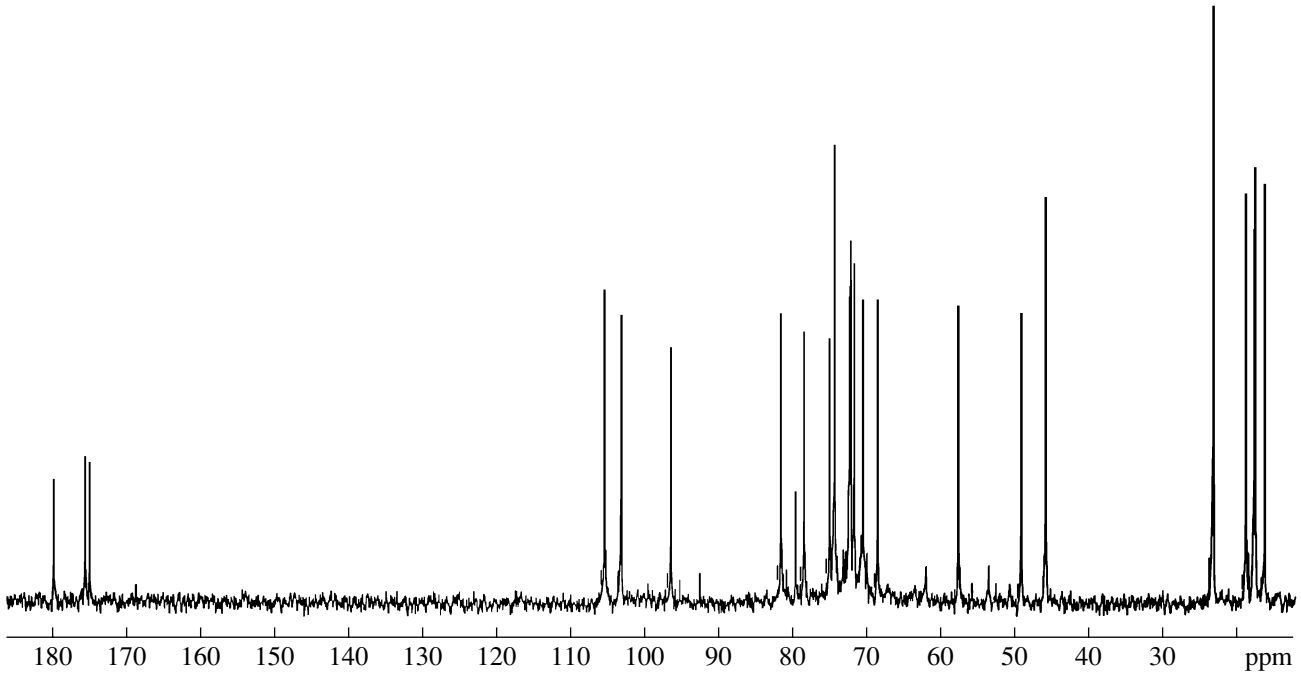

Fig. 3. ^{13}C NMR spectrum of the O-specific polysaccharide of *P. fluorescens* IMV 2366 (biovar III).

amido-2,6-dideoxygalactose (FucNAc), and 3-acetamido-3,6-dideoxyglucose (Qui3NAc) in a ratio of 1.3 : 1 : 0.1 were identified. The small amount of Qui3NAc can be accounted for by the low yield of the N-acyl group from 3,6-dideoxy-3-(3-hydroxy-2,3-dimethyl-5-hydroxyprolylamino)quinovose (Qui3NAcyl) during acid hydrolysis.

The GLC analysis of acetylated (R)-2-octyl glycosides showed that rhamnose had the L-configuration and FucN had the D-configuration. The D-configuration of Qui3N was determined on the basis of the effects of glycosylation in the polysaccharide ^{13}C NMR spectrum. These and other, more detailed, data on the structural analysis of the repeating polysaccharide unit are highlighted in a work published by us earlier [15]. Based on the analysis of all the data obtained, it was established that the repeating O-specific polysaccharide unit of *P. fluorescens* IMV 2366 (biovar III) has the following structure:

⟶ 2)-β-D-Qui*p*3NAcy1-(1 ⟶ 3)-α-L-Rha*p*-(1 ⟶ 3)-α-D-Fus*p*NAc-(1 ⟶.

Table 4. Chemical shifts (δ, ppm) in the ^{13}C NMR spectrum of the O-specific polysaccharide of *P. fluorescens* IMV 2366 (biovar III)

Component	Monosaccharide carbon atoms					
	C-1	C-2	C-3	C-4	C-5	C-6
⟶ 2)-β-D-Qui*p*3Nacy1-(1 ⟶	105.8	75.1	57.9	74.4	74.5	18.4
⟶ 3)-α-L-Rha*p*-(1 ⟶	103.6	71.8	81.6	72.3	70.6	18.1
⟶ 3)-α-D-Fuc*p*NAC-(1 ⟶	96.7	49.4	78.5	72.5	68.7	16.9

Component	Acyl group carbon atoms						
	C-1	C-2	C-3	C-4	C-5	CH_3-2	C_3-3
⟶ 2)-β-D-Qui*p*3Nacy1-(1 ⟶	176.0	79.6	72.3	46.2	180.3	23.9	19.4
	175.7*	**78.2**	**71.6**	**45.8**	**179.7**	**23.2**	**18.4**
⟶ 3)-α-L-Rha*p*-(1 ⟶							
⟶ 3)-α-D-Fuc*p*NAC-(1 ⟶	175.3	23.7					

* Data from the spectrum of the 3-hydroxy-2,3-dimethyl-5-hydroxyprolylamino group of *P. fluorescens* 361 [15].

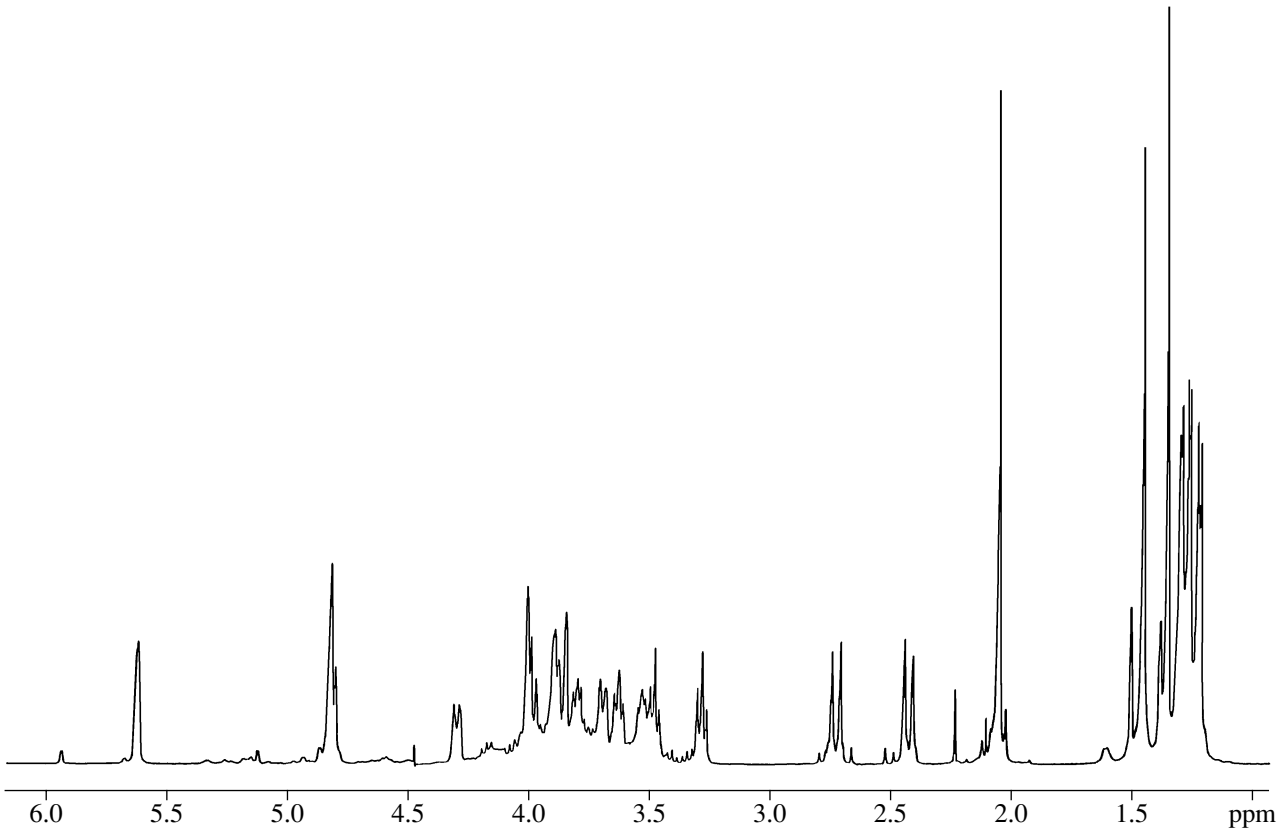

Fig. 4. ^{1}H NMR spectrum of the O-specific polysaccharide of *P. fluorescens* IMV 2366 (biovar III).

Thus, the LPS of *P. fluorescens* IMV 2366 (biovar III) has a structure common to all gram-negative bacteria that is based on the principle of the presence of three different structural portions in the macromolecule: lipid A, the core oligosaccharide, and the O-specific polysaccharide chain. The R- and S-types of LPS molecules were present in the initial pool of the LPS preparation as in the *P. fluorescens* strains studied earlier.

In lipid A of the strain studied, the hydrophilic portion components (glucosamine, phosphoethanolamine, and phosphorus), as well as a set of fatty acids (3-hydroxydecanoic, 2-hydroxydodecanoic, 3-hydroxydodecanoic, and dodecanoic), characteristic of lipid A of the typical representatives of *Pseudomonas* bacteria were found. Lipid A of the strain studied did not differ in the structure of the hydrophilic portion from lipid A of the strains studied earlier, and, thus, it has the same structural organization as the LPS lipid A of *P. fluorescens* bacteria belonging to biovars I and II.

The LPS core oligosaccharide of *P. fluorescens* IMV 2366 differed in part from other core oligosaccharides studied earlier.

The structure of the repeating unit of the LPS O-specific polysaccharide of *P. fluorescens* IMV 2366 is unique. Despite its being made up of the monosaccharides revealed earlier in the LPS O-specific polysaccharides of *P. fluorescens*, the *N*-bonded 3-hydroxy-2,3-dimethyl-5-hydroxyprolyl group, a rarely occurring substitute of noncarbohydrate nature, enters into its composition. Previously, it was found in the O-polysac-

Table 5. Chemical shifts (δ, ppm) in the ^{1}H NMR spectrum of the O-specific polysaccharide of *P. fluorescens* IMV 2366 (biovar III)

Component	Monosaccharide protons						Acyl group protons				
	H-1	H-2	H-3	H-4	H-5	H-6	H-2	H-4a	H-4c	CH_3-2	CH_3-3
⟶ 2)-β-D-Qui*p*3Nacy1-(1 ⟶	4.81	3.62	3.99	3.27	3.53	1.30		2.42	2.71	1.36	1.47
⟶ 3)-α-L-Rha*p*-(1 ⟶	4.82	4.00	3.88	3.47	3.79	1.23					
⟶ 3)-α-D-Fuc*p*NAC-(1 ⟶	5.62	4.30	3.69	3.84	3.89	1.27	2.05				

charide of the unclassified *P. fluorescens* 361 strain. In the O-specific polysaccharide of *Vibrio cholerae* and *Vibrio anguillarum*, the derivatives of pyroglutamic acid—the 3-hydroxy-3-methyl-5-hydroxyprolyl and 2,4-dihydroxy-3,3,4-trimethyl-5-hydroxyprolyl groups, respectively—were revealed [15]. It is noteworthy that all these groups were the substitutes of 6-deoxyamino sugars, which often include unusual *N*-acyl substitutes. Proceeding from the fact that the LPS O-chains of *P. fluorescens* IMV 2125 also include rhamnose and fucosamine [16] but do not enter into cross reactions with the LPS of the strain studied in serological tests, it may be concluded that the above monosaccharides are not immunodominant in the O-chain. The absence of serological relations between the strain studied and *P. fluorescens* IMV 2125 (biovar III), as well as the strains representing the other *P. fluorescens* biovars, correlates well with the known heterogeneity of the strains of the taxon studied, which is reflected in their division into biovars and heterogeneity within the biovars.

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