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EXPERIMENTAL ARTICLES

Chemical and Biological Characterization of Lipopolysaccharides from the *Pseudomonas syringae* pv. *maculicola* IMV 381 Collection Culture and Its Dissociants

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Abstract—Lipopolysaccharides (LPS) were isolated from the crude bacterial mass of the *Pseudomonas syringae* pv. *maculicola* IMV 381 collection culture and its virulent and avirulent subcultures isolated earlier from the heterogeneous collection culture due to its natural variability during long-term storage. The composition, immunochemical properties, and certain parameters of the biological activity of the LPS preparations obtained were studied. The structural parts of the LPS macromolecule—lipid A, the core oligosaccharide, and O-specific polysaccharide (OPS)—were isolated and characterized. The following fatty acids were identified in the lipid A composition of all cultures: 3-OH-$C_{10:0}$, $C_{12:0}$, 2-OH-$C_{12:0}$, 3-OH-$C_{12:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$. Glucosamine (GlcN), ethanolamine (EtN), phosphoethanolamine (EtN-P), and phosphorus (P) were revealed in the hydrophilic portion of the macromolecule. In the core portion of the LPS macromolecule, glucose (Glc), rhamnose (Rha), GlcN, galactosamine (GalN), 2-keto-3-deoxyoctulosonic acid (KDO), alanine (Ala), and P were found. The peculiarities of the structure of LPS isolated from the stable collection culture (LPS_{stab}) and its virulent (LPS_{vir}) and avirulent (LPS_{avir}) subcultures were studied. LPS_{vir} and LPS_{avir} were identical in the monosaccharide composition and contained as the main components L-rhamnose (L-Rha) and 3-acetamido-3,6-dideoxy-D-galactose (D-Fuc3NAc), like LPS_{stab}, studied earlier. The NMR spectra of LPS_{vir} were identical to the spectra of LPS_{stab}, whose O-chain repeating unit structure was studied by us earlier, whereas LPS_{avir} differed from LPS_{vir} in the NMR spectrum and was identified by us as the SR form. LPS_{avir} was serologically identical to LPS_{stab} and LPS_{vir}. Hence, the degree of polymerism of the LPS O-chain of *P. syringae* pv. *maculicola* IMV 381 is the main virulence factor in infected model plants. Serological relationships were studied between *P. syringae* pv. *maculicola* IMV 381 and the strains of other pathovars with structurally similar LPS.

Key words: Pseudomonas syringae, dissociation, virulence, lipopolysaccharide, O-specific polysaccharide, core oligosaccharide, lipid A, composition, structure, biological activity.

When studying the culture of the previously stable collection strain *P. syringae* pv. *maculicola* IMV 381, we revealed the heterogeneity of its microbial population [1]. Isolated in the individual state and differing in the morphology of colonies, the subcultures, upon testing of their properties in indicator plants, were identified as virulent and avirulent. Despite the differences revealed, both subcultures were agglutinated in equal or very close titers with O- and OH-antiserums against the homologous and heterologous subcultures, as well as with the O- and OH-antiserums obtained by us earlier (before dissociation was manifest) against the *P. syringae* pv. *maculicola* IMV 381 culture. Thus, the previously stable culture and its virulent and avirulent subcultures are serologically identical.

Lipopolysaccharides (LPS) are the main cell surface component of gram-negative bacteria and determine their O-specificity. These biopolymers are one of the virulence factors. They mediate many biological activities in various biological systems. These activities may correlate with the length of the LPS O-chain [2].

In this work, the composition, structural peculiarities, and certain biological properties of LPS from the stable collection culture and its virulent and avirulent subcultures are described in detail in the comparative aspect in order to clarify problems of the systematics and taxonomy of the species *P. syringae* and to examine the influence of LPS on the virulence of *P. syringae* pv. *maculicola* IMV 381.

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MATERIALS AND METHODS

The object of study was strain *P. syringae* pv. *maculicola* IMV 381 from the Collection of Viable Cultures of the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. The conditions for culture storage and maintenance, cultivation and accumulation of the bacterial mass, and isolation of unpurified preparations are described by Yakovleva *et al.* [1].

The lipopolysaccharide–protein complexes isolated from the microbial cells by extraction with 0.85% NaCl were purified by repeated (4 h × 3) ultracentrifugation at 105000 *g*.

The carbohydrate, protein, phosphorus, 2-keto-3-deoxyoctulosonic acid (KDO), and heptose contents were determined as described by Zdorovenko *et al.* [3].

Lipopolysaccharide (LPS) cleavage was performed with 1% acetic acid at 100°C for 1.5 h [3]. Lipid A precipitate was separated by centrifugation. The water-soluble fraction was separated in a 65 × 1.6 cm column with Sephadex G-50 gel (Pharmacia, Sweden) in 0.05 M pyridine–acetate buffer (pH 4.5) by controlling elution with the reaction with phenol and sulfuric acid. High-molecular O-specific polysaccharide (OPS) and core oligosaccharide fractions were obtained as a result.

The analysis of the monosaccharide composition was performed by hydrolyzing polysaccharide with 2 M trifluoroacetic acid (121°C, 2 h). After concentrating the hydrolysate by evaporation, monosaccharides were converted to polyol acetates (by reduction with sodium borane and acetylation) and assayed using gas–liquid chromatography (GLC) on a Hewlett-Packard 5880 chromatograph furnished with a capillary column with the Ultra 2 stationary phase within the temperature gradient 150 ⟶ 290°C (10°C/min) and GLC–mass spectrometry on a Hewlett-Packard 5989 chromatograph–mass spectrometer equipped with a capillary column with the stationary phase HP-1.

The NMR spectra were recorded using Bruker DRX-500 spectrometers (Germany) in 99.96% D_2O at 53°C (acetone was used as the internal standard; δ_H 2.225 ppm; δ_C 31.45 ppm). The samples were lyophilized twice from D_2O. Two-dimensional spectra were recorded using the standard mathematical software (Bruker); the software XWINNMR, version 2.1, was used for data collection and processing. The mixing time in the TOCSY and NOESY experiments was 120 and 200 ms, respectively.

Immune sera were prepared and the precipitation reactions, double diffusion in agar according to Ouchterlony, immunoelectrophoresis, and immunoenzyme assay (IEA) were performed as described in [1, 3, 4].

Amino compounds (amino sugars, ethanolamine, phosphoethanolamine, and amino acids) were determined after the acid hydrolysis of preparations in an amino acid analyzer [4]. The lipid A fatty acid composition was determined after methanolysis of the samples using the methods of GLC and GLC–mass spectrometry as described in [3, 4].

The LPS toxicity and its antitumor activity were studied with white mongrel mice bred in the experimental laboratories of the Kavetskii Institute of Experimental Pathology, Oncology, and Radiobiology, National Academy of Sciences of Ukraine. The toxicity was determined by single injections of the substance into the abdominal cavity of test animals, and the time of the animals' death was recorded.

A transplantable mouse tumor—Ehrlich's ascitic carcinoma—was used as a tumor growth model. The animals were inoculated with the tumor (2.5×10^5 tumor cells per animal). Treatment was begun on the day following the tumor transplantation. The preparation was injected twice: on the following day and six days after transplanting the tumor. The cumulative dose of the preparation constituted 20 mg/kg of the animal's body mass. The preparation was dissolved in saline. The antitumor activity was assessed by changes in the life span of the test mice compared with the untreated control mice.

The cytotoxic activity was studied in in vitro experiments using lymphocytes obtained from the peripheral lymph nodes of white mongrel mice and in a HeLa cell line culture (human cervical carcinoma) in the following way. The lymphocytes obtained by the mechanical destruction of mouse peripheral lymph nodes were washed twice with Hanks' solution, and the cells were resuspended to 10 million/ml and incubated for 20 h at 37°C in the nutrient medium 199 in the presence of the LPS preparation at a concentration of 50 μg/ml, which was calculated based on the data on the preparation toxicity determined in the experiments in vivo. The incubation was carried out in a standard plastic plate. Upon completion of incubation, the lymphocytes were washed twice with Hanks' solution at pH 7.4 and stained for 7 min with 0.1% trypan blue in 0.9% NaCl. The proportion of viable cells unstained with trypan blue was determined by microscopy in a Goryaev chamber.

When determining cytotoxicity in the HeLa cell culture, the cells were grown in Rose's perfusion chambers in a laminar box at 37°C. The nutrient medium contained 45% Eagle's medium (with glutamine dissolved extempore), 45% 0.5% lactalbumin hydrolysate in Hanks' solution, 10% bovine blood sera, and antibiotics (gentamicin + streptomycin). HeLa cells were plated at a rate of about 100 cells per mm^2 of glass in the chamber; the preparation was introduced 24 h after plating the culture. The kinetics of culture growth both in the control and in the experimental variants was studied by determination of the cell monolayer density as a function of the cultivation time. Every value is the arithmetic mean of three measurements in three visual fields of the microscope within one chamber.

The tumor necrosis factor (TNF) was determined as described in [5].

Table 1. General characteristics of the LPS preparations from the collection culture of *P. syringae* pv. *maculicola* IMV 381 and its different subcultures

Component	Preparation		
	LPS_{stab}	LPS_{vir}	LPS_{avir}
	% of the dry matter weight		
Yield	7.2	10.0	1.0
Carbohydrates	57.0	+	+
Lipid A	49.0	+	+
Protein	3.2	+	+
Phosphorus	+	2.5	4.75
Heptoses	+	0.4	traces
2-keto-3-deoxyoctulosonic acid (KDO)	+	+	+
Glucosamine	2.6	0.6	5.4
Galactosamine	1.2	1.7	2.3
Ethanolamine	0.7	1.0	3.6
Phosphoethanolamine	0.7	0.6	3.0
Alanine	0.3	4.8	3.5
	% of the total content (peak areas, GLC)		
Rhamnose	62.5	14.0	41.1
3-amino-3,6-dideoxy-D-galactose	30.0	7.2	14.1
Glucose	7.5	3.1	13.3

Note: "+" denotes the presence of a compound.

Electrophoresis in PAAG in the system with sodium dodecyl sulfate (SDS) was performed according to the technique described in [6]. The LPS preparations (1–2 mg/ml) were dissolved in the buffer, pH 8.8 (0.5 M Tris–HCl with 2-mercaptoethanol, 10% SDS, 20% glycerol, and 0.001% bromophenol blue), and boiled for 10 min, after which 40 μl was applied on the gel. Electrophoresis was performed in 5% acrylamide concentrating and 12% acrylamide separating gels at a constant current strength of 30 mA. The gels were stained with silver nitrate.

RESULTS AND DISCUSSION

The LPS_{stab} isolated earlier from the crude microbial mass of the collection culture of *P. syringae* pv. *maculicola* IMV 381 by a sparing method (washout with 0.85% NaCl) and purified by dialysis and repeated ultracentrifugation contained carbohydrates (determined by the reaction with phenol and sulfuric acid) and protein (determined by the Lowry method) (Table 1). After methanolysis, GLC revealed in the preparation fatty acids, including hydroxy acids—characteristic lipid A components (Fig. 1). The components listed in Table 1 were identified in the composition of the preparation studied with the GLC method, specific reactions to KDO and heptoses, and ion-exchange chromatography in an amino acid analyzer.

These data show that rhamnose is present in the composition of the preparation studied as the main neutral sugar, as in the representatives of this group of bacteria studied earlier [7]. KDO and heptoses, characteristic components of the lipopolysaccharide molecule, were also revealed.

LPS_{stab} revealed serological activity with the homologous O-serum obtained by immunizing rabbits with microbial cells killed by heating (2.5 h, 100°C). In the precipitation and passive hemagglutination (PHAR) reactions, the titers were 1 : 100000 and 1 : 640, respectively; two different precipitation lines were revealed in the reactions of double diffusion in agar (DDA) and immunoelectrophoresis. It was determined in direct and cross agglutination, PHAR, and DDA reactions with polyvalent native and partially adsorbed sera that the O-antigen formula of the strain studied contains no less than three differently expressed antigenic determinants. According to the classification scheme [8], strain *P. syringae* pv. *maculicola* IMV 381 belongs to serogroup I. In direct and cross agglutination reactions with polyvalent and partially adsorbed sera, serological interrelationships were determined between the strain studied and the strains of other pathovars (*P. syringae* pv. *atrofaciens* IMV 8281, pv. *syringae* IMV 281, pv. *syringae* (*holci*) IMV 8300, and pv. *syringae* (*populi*) IMV 460) entering into the composition of serogroup I, as well as strains *P. syringae* pv. *tabaci* IMV 223 and

pv. *tomato* IMV 140 R, assigned to serogroup VII in the above-mentioned classification scheme.

The LPS_{stab} preparation isolated by us revealed toxicity in white mongrel mice, LD_{50} and LD_{100} constituting 15–20 and 25–50 mg/kg, respectively. The preparation also exerted a cytotoxic effect on the lymphocytes of normal mice. The survival rate was 69.2%, compared with a survival rate of 89.7% observed in the control mice.

It is known from the literature [9] that LPS may show antitumor activity. The study of the antitumor activity of LPS_{stab} in vitro using the HeLa cell line (human cervical carcinoma) as a model showed it to have a mild cytotoxic action (Table 2). To study the antitumor activity in vivo, the influence of LPS_{stab} on the growth of Ehrlich's ascitic carcinoma in mice was assessed (Table 3). These data show that not only did the LPS of the strain studied not exhibit antitumor activity in relation to Ehrlich's mouse carcinoma, but, on the contrary, it actively stimulated the growth of this tumor.

Thus, different influences of the LPS of the strain studied on tumor growth were determined in the experiments in vitro and in vivo. The differences revealed seem to be explained by the fact that the studies were conducted with different types of tumor cells. The fact of a different relation of the same LPS to different tumors is known in the literature [9] and has been observed by us repeatedly (unpublished data).

The preparations isolated from the virulent (LPS_{vir}) and avirulent (LPS_{avir}) subcultures of the strain studied contained all the components identified in the composition of LPS_{stab} (Table 1). The analysis of the data obtained shows that the avirulent subculture considerably differs from other subcultures in LPS yield when it is extracted with saline. In this case, LPS_{avir} contained comparatively more glucosamine, galactosamine, ethanolamine, phosphoethanolamine, and glucose, which are usually the characteristic components of the LPS core oligosaccharide, which may give evidence of an increased specific content of the latter in this preparation.

LPS_{vir} and LPS_{avir}, like LPS_{stab}, revealed biological activity in relation to mammalian cells. As seen from the data on testing of the blood of 12 donors, the LPS_{vir} and LPS_{avir} preparations were able to stimulate TNF (tumor necrosis factor) production by human peripheral blood cells, as well as to stimulate the proliferative response of these cells, but the degree of the effect was different (Table 4). The activity revealed was lower than

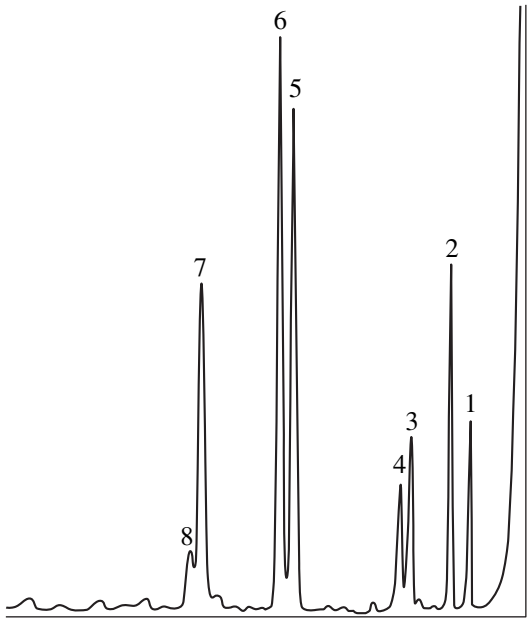


Fig. 1. GLC profiles of methyl ethers of the LPS lipid A fatty acids of *P. syringae* pv. *maculicola* IMV 381. Fatty acids: (1) 3-OH-$C_{10:0}$, (2) $C_{12:0}$, (3) 2-OH-$C_{12:0}$, (4) 3-OH-$C_{12:0}$, (5) $C_{16:1}$, (6) $C_{16:0}$, (7) $C_{18:1}$, and (8) $C_{18:0}$.

the activity manifested by the classical endotoxin, *E. coli* LPS, used by us as a positive control. The LPS from the other bacterial strains of the genus *Pseudomonas* also revealed a comparatively low biological activity in different tests [5], which is clearly explained by the specific features of the composition and structure of lipid A of these bacteria, which differs from lipid A of enterobacteria and is similar in all representatives of the genus. Different in biological activity, LPS_{vir} and LPS_{avir} are similar in composition (Table 1). It was therefore concluded that the biological activity of these preparations is determined by the structural peculiarities of the LPS macromolecule. LPS_{vir} and LPS_{avir}, like LPS_{stab}, revealed serological activity in the precipitation reaction with homologous polyvalent O-sera (titer 1 : 10000) prepared by immunizing rabbits with cells of the virulent and avirulent subcultures killed by heating. The serological identity of the LPS isolated from the collection culture and from its virulent and avirulent subcultures was determined in cross agglutination reactions of microbial cells, as well as in cross precipitation reactions of the LPS preparations with polyvalent O-sera. The serological identity of the preparations tes-

Table 2. Influence of LPS_{stab} on the growth of the HeLa cell culture (human cervical carcinoma)

Preparation (1 mg/ml)	Incubation time, days, and cell numbers/mm^2					
	0	1	2	3	4	5
Control	82	86	114	167	180	152
LPS_{stab}	81	88	122	118	110	106

Table 3. Influence of LPS_{stab} on the growth of Ehrlich's ascitic carcinoma in mice

Preparation (cumulative dose 20 mg/ml)	Average life span of the animals (days)	Inhibition or stimulation (+) of the tumor growth, %
Control	14.8 ± 0.9	–
LPS_{stab}	12.0 ± 2.1	+19

Table 4. LPS-activated TNF production and the proliferative response of human peripheral blood mononuclear cells ($n = 12$)

Preparation (10 μg/ml)	Biological activity	
	TNF production (pg/ml ± *SD*)	Proliferative response (*Cpm* ± *SD*)
E. coli LPS	2675 ± 921	12173 ± 1914
LPS_{vir}	1247 ± 405	2178 ± 724
LPS_{avir}	825 ± 422	3130 ± 684

tified to the structural identity or closeness of the O-specific polysaccharide chains of these LPS. Considering the similarity of the composition and the serological identity and, hence, the similarity of the O-chain structure, the differences in biological activity may be determined by the specific features of the structural organization of the LPS macromolecule, which may also be responsible for differences in the character of growth of the virulent and avirulent subcultures [1].

In order to answer these questions, comparative physicochemical (PAAG electrophoresis with sodium dodecyl sulfate, gel chromatography of the carbohydrate portion of the macromolecule on Sephadex G-50) and structural studies of LPS_{stab}, LPS_{vir}, and LPS_{avir} were conducted.

The lipid A, core oligosaccharide, and O-specific polysaccharide fractions were isolated in the individual state from each LPS by its mild acid degradation followed by chromatography on the Sephadex G-50 gel (Fig. 2). As seen from the elution curves shown in the figure, the ratio of the isolated fractions indicates that the OPS fraction (peak I), i.e., S-LPS, predominates in the preparation from the collection culture (Fig. 2a), which is also characteristic of the *P. syringae* strains studied by us earlier [7]. The elution curve of degraded LPS_{vir} is of a similar character (Fig. 2b): compared with LPS_{stab}, only an insignificantly increased specific content of the core oligosaccharide fraction (peak II) may be noted. The elution curve of degraded LPS_{avir} (Fig. 2c) does significantly differ from the others in a low specific content of the OPS fraction (peak I) and the presence of peaks Ia and Ib, which, by mobility, may be identified as those representing the SR-LPS fraction [6].

The gel filtration data are consistent with the results obtained when the LPS_{stab}, LPS_{vir}, and LPS_{avir} preparations were studied by the electrophoresis in PAAG method. Here (Fig. 3), as in the case of gel filtration, LPS_{avir}, contrary to LPS_{stab} and LPS_{vir}, has a poorly defined high-molecular-weight zone (band I), which may accounted for by S-LPS, and a well-defined heterogeneous medium-molecular-weight zone is present (bands Ia and Ib), which may be identified as heterogeneous SR-LPS. Band II in all preparations is likely to represent R-LPS.

Thus, LPS_{avir}, according to the gel filtration and electrophoresis in PAAG data, may be identified as SR-LPS containing only an insignificant admixture of S-LPS, which is explained by the difficulty of building up the biomass of the avirulent subculture without an admixture of microbial cells of the virulent subculture in it due to the increased instability of dissociant cultures. It is known [6] that SR-LPS differs from S-LPS only in the degree of the O-chain polymerism and does not differ serologically. The identification of LPS_{avir} as SR-LPS agrees well with the serological identity of the LPS preparations determined by us.

The following fatty acids were identified as the components of the lipid A fraction from LPS_{stab} using the methods described in [3, 4]: 3-OH-$C_{10:0}$, $C_{12:0}$, 2-OH-$C_{12:0}$, 3-OH-$C_{12:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$ in the ratio (% of the peak area sum, GLC) 6.9 : 12.5 : 6.3 : 4.5 : 22.8 : 25.9 : 17.7 : 3.0, respectively. It follows from these data that *P. syringae* pv. *maculicola* IMV 381 is not different from the *P. syringae* strains studied earlier [3, 4, 7], as well as from *P. fluorescens* strains [10], in the fatty acid composition. In addition to the typical, and common for lipid A of representatives of the genus *Pseudomonas* [4] fatty acids identified as lipid A components at the structural level [11], such as 3-OH-$C_{10:0}$, $C_{12:0}$, 2-OH-$C_{12:0}$, and 3-OH-$C_{12:0}$, the composition of all the lipids A studied, including lipid A of strain IMV 381, reveals the following fatty acids: $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$. The possible causes of their presence are discussed in detail in [3, 4, 10]. Glucosamine, ethanolamine, and phosphoethanolamine in a ratio of 5.4 : 2.4 : 2.7 (% of the peak area sum, amino acid analyzer), respectively, as well as a number of amino acids, were identified as the components of the hydrophilic portion of the lipid A macromolecule of the culture studied. As evidenced by the GLC data, the preparation contained 1.1% phosphorus and did not contain an admixture of neutral sugars.

The same spectrum of fatty acids as in LPS_{stab} was identified in the composition of the LPS_{vir} and LPS_{avir} lipid A, the cultures differing in the specific content of the fatty acids typical of the *Pseudomonas* lipid A (3-OH-$C_{10:0}$, $C_{12:0}$, 2-OH-$C_{12:0}$, and 3-OH-$C_{12:0}$). Their content was (% of the peak area sum) 37, 18.1, and 45.8% for LPS_{stab}, LPS_{vir}, and LPS_{avir}, respectively.

The components listed in Table 5 were identified in the composition of the LPS_{stab} core oligosaccharide fraction obtained upon gel filtration of the polysaccharide on the column with Sephadex. Neutral sugars were identified by the GLC method in the form of polyol acetates; amino

compounds, by using the amino acid analyzer. Specific reactions were used for determining heptoses, KDO, and phosphorus. The neutral sugars rhamnose and glucose revealed by us in the composition of the LPS_{stab} core oligosaccharide are the characteristic components of this portion of the LPS macromolecule of the *Pseudomonas* representatives, in particular, *P. syringae* [3, 4, 7, 11]. The same monosaccharides were identified by us at the structural level in the core of strain *P. syringae* pv. *phaseolicola* GSPB 711 [12]. However, of the neutral sugars, only glucose was described in the LPS core composition of the hitherto only *Pseudomonas fluorescens* strain whose LPS core has been characterized at the structural level [13]. Therefore, it seemed unlikely that differences in the virulence of *P. syringae* pv. *maculicola* IMV 381 dissociants are determined by differences in the core oligosaccharide structure.

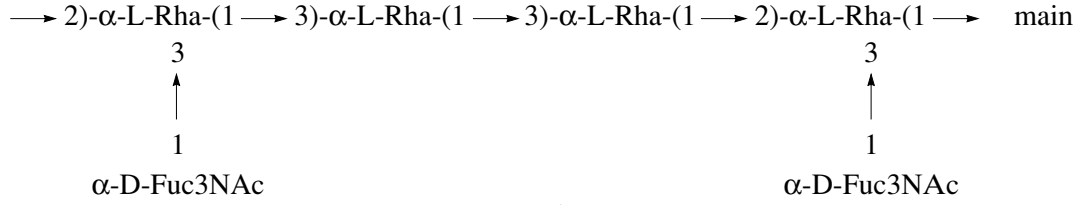
The structural analysis of the OPS fractions was then performed. The acid hydrolysis of polysaccharide from LPS_{stab} with 2 M trifluoroacetic acid with a subsequent assay of monosaccharides in the form of polyol acetates using the GLC method led to the identification of rhamnose (Rha) and 3-amino-3,6-dideoxy-D-galactose (D-Fuc3N).

The ^{13}C NMR (Fig. 4a) and ^{1}H NMR (Fig. 4b) polysaccharide spectra contained signals of different intensity, which evidenced the absence of strict regularity of its structure. The spectra contained the signals of a number of anomeric atoms (δ_C 96.1–103.3; δ_H 5.03–5.27) and the methyl groups of 6-deoxy sugars (δ_C 16.5 for Fuc3NAc and 18.0–18.2 for Rha; δ_H 1.17–1.18 for D-Fuc3NAc and 1.25–1.34 for Rha in a ratio of 1 : 2.5), and of *N*-acetyl groups (δ_H 2.04–2.05; δ_C 23.4 (Me) and 175.6 (CO)). It is concluded on the basis of the data shown that the polysaccharide is heterogeneous and mainly consists of repeating hexasaccharide units containing four L-Rha residues and two D-Fuc3NAc residues.

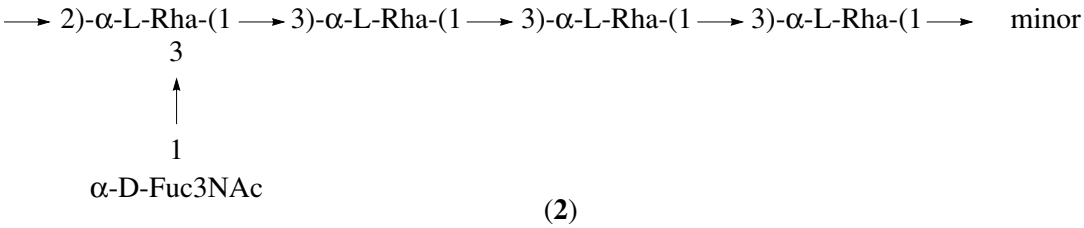
Table 5. Component structure of the LPS_{stab} core oligosaccharide fraction

Component	Content
	% (peak area, GLC)
Rhamnose	41.5
Glucose	59.5
	% of the dry matter weight
Glucosamine	6.3
Galactosamine	5.3
KDO	traces
Phosphorus	7.3
Alanine	3.5
Heptoses	traces

The more detailed analysis of OPS using NMR spectroscopy, as described in detail in [14] by the example of OPS from the LPS of *P. syringae* pv. *tomato* GSPB 483, showed that the LPS OPS from the collection strain *P. syringae* pv. *maculicola* IMV 381 is made up of two types of repeating units with the following structure:



(**1**)



(**2**)

That is the O-chains of the LPS of the strain studied contain hexa- and pentasaccharide repeating units including four L-Rha residues and either one or two D-Fuc3NAc residues. Thus, the OPS of *P. syringae* pv. *maculicola* IMV 381 has a structure common for all LPS of the hitherto studied *P. syringae* strains: it contains linear rhamnan as one of the O-chain constituents. This gives evidence in favor of the taxonomic significance of this characteristic. The major and minor units are present in a ratio of 2 : 1. The heterogeneity of the LPS O-chain is characteristic of the *P. syringae* strains assigned to serogroups I, IV, V, and VII [4, 11] and *P. fluorescens* strains [10, 15]. Other authors observed it in strains of *Ralstonia solanacearum* and *B. cepacia* [16]. The structure of the LPS O-chain of the strain IMV 381 described by us in this work was also identi-

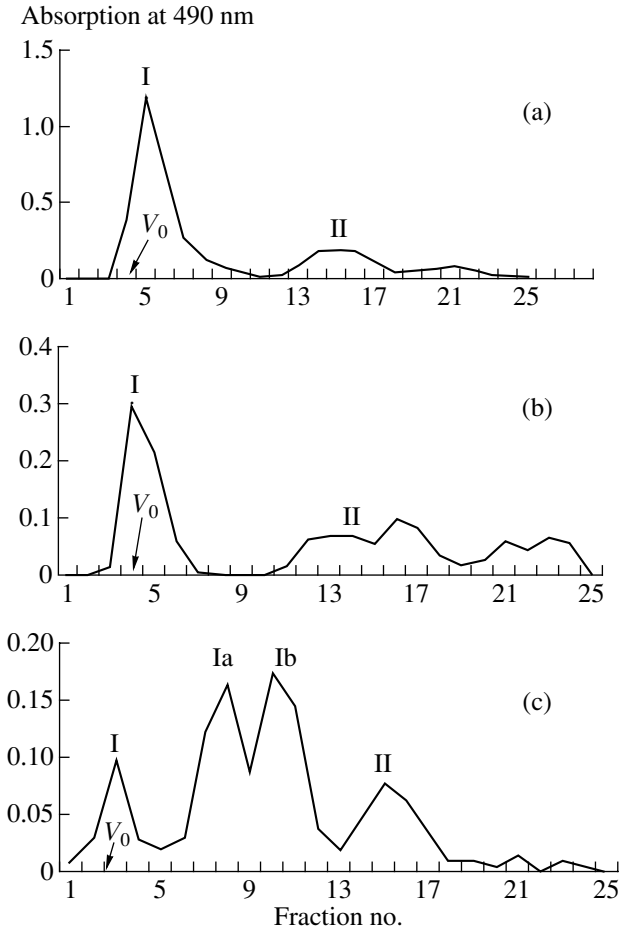


Fig. 2. Elution profiles of the gel filtration of the (a) LPS_{stab}, (b) LPS_{vir}, and (c) LPS_{avir} polysaccharides of *P. syringae* pv. *maculicola* IMV 381 on a column with Sephadex G-50: (I–Ib) O-specific polysaccharide fraction; (II) core oligosaccharide fraction; the free volume V_0 = 98 ml; 1–25 are 10-ml fractions.

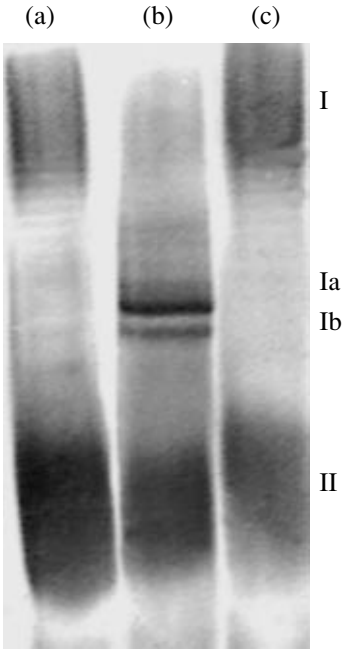


Fig. 3. PAAG electrophoresis of the (a) LPS_{vir}, (b) LPS_{avir}, and (c) LPS_{stab} of *P. syringae* pv. *maculicola* IMV 381. I, Ia and Ib, and II are the carbohydrate bands representing the S, SR, and R LPS zones, respectively, in the complex LPS preparations from the collection culture and its subcultures.

fied in *P. syringae* pv. *tomato* 483 and *P. syringae* pv. *syringae* (*populi*) IMV 460 based on the identity of the NMR spectra. Moreover, it is structurally similar to the LPS of a number of *P. syringae* strains (Table 6). These data indicate that, in the structure of the O-chain repeating unit, the LPS from the strains of serogroups I and VII show this or that extent of structural similarity to the LPS of *P. syringae* pv. *maculicola* IMV 381.

Immunochemical studies with IEA showed a high degree of affinity of the strain studied with strains IMV 8281, IMV 8300, and IMV 281, whose LPS O-chains, which are heterogeneous in structure, incorporate the repeating unit with the structure (**2**) identified by us in the composition of OPS from LPS_{stab}. Thus, LPS_{stab} at a concentration of 1.56–25 μg/ml actively (by 56–100%) inhibited the IEA reaction in the system containing O-serum against strain IMV 281 cells + LPS of this strain, which may be explained by the presence of the common repeating unit (Table 6) with the structure (**2**) in the OPS composition of both strains.

In the case of structural similarity but not identity, as in the case of IMV 223 LPS (Table 6, structure (**7**)), only slight serological affinity was observed. The LPS of IMV 381 at a concentration of 1.56–200 μg/ml inhibited the IEA reaction in the system O-serum against strain IMV 223 microbial cells + strain 223 LPS only by 0–13%. Such a weak interrelationship may be due to factors other than the antigen determinants present in the O-chain.

Hereinafter, the structural analysis of OPS in the composition of LPS_{vir} and LPS_{avir} was performed.

Acid hydrolysis of OPS from LPS_{vir} allowed the identification of Rha and D-Fuc3N, as in the case of LPS_{stab}.

The analysis of the ^{13}C NMR and ^{1}H NMR spectra of the OPS from LPS_{vir} showed them to be identical to those described above (Figs. 4a, 4b) for OPS from LPS_{stab}. Thus, based on these data, the LPS O-polysaccharide of the virulent subculture of *P. syringae* pv. *maculicola* IMV 381 is made up of repeating penta- and hexasaccharide units containing four L-Rha residues and one or two D-Fuc3NAc residues.

Acid hydrolysis of the predominant Ia fraction (Fig. 3) from LPS_{avir} led, as in the case of LPS_{stab} and LPS_{vir}, to the identification of Rha and D-Fuc3N. However, the analysis of the NMR spectra of this

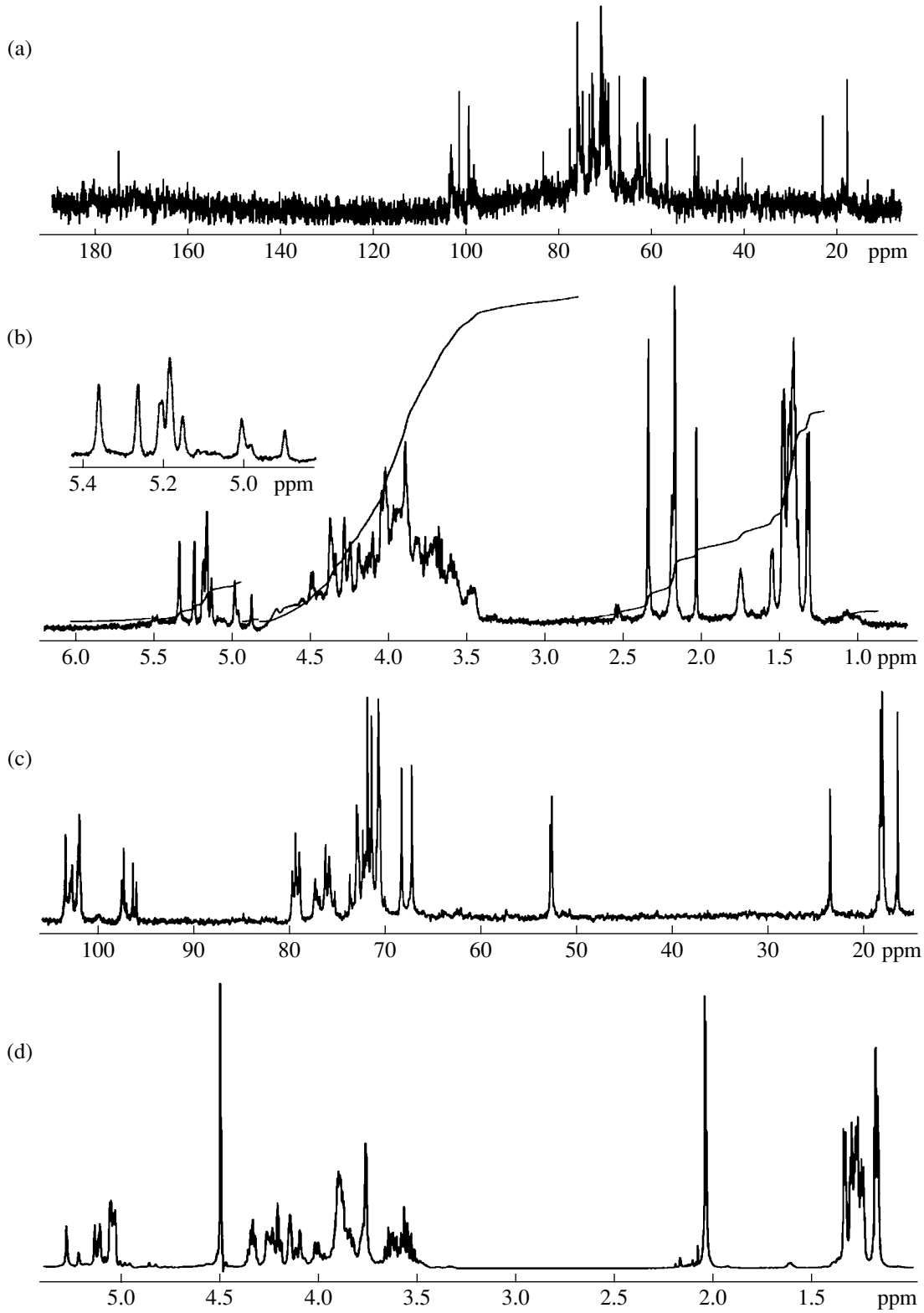


Fig. 4. (a) ^{13}C NMR and (b) ^{1}H NMR spectra of the OPS of the collection culture and (c) ^{13}C NMR and (d) ^{1}H NMR spectra of the OPS of the avirulent subculture of *P. syringae* pv. *maculicola* IMV 381.

Table 6. Structure of the LPS O-polysaccharides of *P. syringae* strains

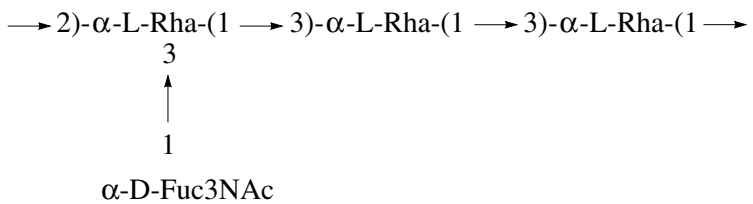
Pathovar and strain	Structure of the repeating unit	Serogroup
atrofaciens IMV 8281	→ 2)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(3)**	I
syringae (*holci*) IMV 8300	→ 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(2)**	
syringae IMV 281	→ 3)-α-L-Rha*p*-(→ 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(4)**	
garcae 588		
coronafaciens IMV 9030	→ 2)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(3)**	
	→ 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → **(5)**	
	→ 2)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → **(6)**	
tabaci IMV 223	→ 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → └ (2 → 1)- α-D-Fuc*p*3NAc **(7)**	VII
tomato 140 R	→ 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(2)**	
	→ 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-1(→ α-D-Fuc*p*3NAc-(1 → 3) ⌋ **(4)**	

polysaccharide revealed its considerable differences from LPS_{stab} OPS and LPS_{vir} OPS.

The ^{13}C NMR (Fig. 4c) and ^{1}H NMR (Fig. 4d) spectra of the LPS_{avir} OPS (Ia fraction, Fig. 2c) contained the main series of signals, including the signals of four anomeric atoms (δ_C 97.2, 102.3, 103.1, and 103.8; δ_H 5.04, 5.08, 5.14, and 5.24), four methyl groups of 6-deoxy sugars (δ_C 16.5 for Fuc3NAc and 18.0–18.2 for Rha; δ_H 1.18 for D-Fuc3NAc and 1.26–1.34 for Rha), and one *N*-acetyl group ((δ_C 23.4; δ_H 2.04 (Me) and 175.6 (CO)). Thus, the main series in the spectra belongs to a repeating tetrasaccharide unit including three Rha residues and one Fuc3Ac residue.

The study of OPS with two-dimensional NMR spectroscopy, including the experiments $^{1}H,^{1}H$ COSY, TOCSY, NOESY, and $^{1}H,^{13}C$ HSQC, revealed correlations between the signals of the monosaccharide residues, which were similar to the corresponding correlations in the repeating tetrasaccharide unit in the composition of the OPS of *P. syringae* pv. *atrofaciens* IMV 8281, studied earlier [17]. In particular, the NOESY and $^{1}H,^{13}C$ HSQC spectra showed a similar character of monosaccharide substitution and the NOESY spectrum demonstrated the same sequence of monosaccharide residues in the repeating units of both polysaccharides.

Thus, the main polysaccharide present in the LPS_{avir} Ia fraction has the following structure:



(**4**)

As already noted (Table 6), the repeating tetrasaccharide units with such a structure are present as minor units in the OPS of *P. syringae* pv. *atrofaciens* IMV 8281 [17] and as the main ones in the OPS of *P. syringae* pv. *tomato* 140 (R) [18]. However, as distinct from these two polysaccharides, the polysaccharide present in the Ia fraction has a short side chain (two or three repeating units on average). This follows from a relatively high intensity of the signals in the NMR spectra, which may belong to the core oligosaccharide. Furthermore, indistinct signals, which may belong to the pentasaccharide units with the structure (**2**), are present in the NMR spectra. The structural analysis data that suggest the LPS_{avir} OPS to be represented by short chains confirm at the molecular level the results of gel filtration and electrophoresis in PAAG (Figs. 2c, 3b). It is known from the literature [2, 19] that the virulence of a microbial cell correlates with the length of the OPS chain and does not depend on its composition and structure. The results of the comparative studies conducted by us, including the structural analysis data, agree well with the literature data and lead us to suggest that the very weak virulence of the avirulent dissociant, as compared to that of its virulent counterpart [1], is determined by a different degree of polymerism of the OPS chains of their LPS. It also follows from the structural analysis data that the O-chain polymerism influences the LPS-stimulated activity of TNF production by human peripheral blood cells and the proliferative response of these cells (Table 4).

However, LPS_{avir} differs from LPS_{stab} and LPS_{vir} not only in the low O-chain polymerism but also in the structure of the predominant repeating units. According to the data of immunochemical studies, as mentioned above, LPS_{avir} is serologically identical to LPS_{stab} and LPS_{vir}. These facts can only be explained by the serological inertness of structure (**1**) in the heterogeneous OPS from LPS_{stab} and LPS_{vir} due to the substitution of two neighboring L-Rha residues with D-Fuc3NAc residues. This agrees with the data obtained by us when we decoded the chemical nature of the antigenic determinants in the composition of the heterogeneous OPS of *P. fluorescens* IMV 4125 LPS and the OPS of *P. syringae* pv. *tabaci* IMV 223 LPS [20], where a similar substitution of two neighboring L-Rha residues with the lateral residues is observed. Therefore, the serological relationship between LPS_{stab}, LPS_{vir}, and LPS_{avir} may only be mediated by pentasaccharide units, which, along with the NMR spectroscopy data, testifies in favor of their presence in the composition of the Ia fraction. However, the substitution of two neighboring rhamnose residues with the lateral substituents may be connected with the virulent properties of the culture and probably occurs at the later stages of the O-chain biosynthesis as its modification in order to adjust to the environmental conditions.

Thus, the LPS of the *P. syringae* pv. *maculicola* IMV 381 collection strain isolated with a rare sparing method (washing out with saline) has a usual macromolecular organization. In the complex preparation, the S-form molecules predominate. The LPS exerts a low-toxicity effect on mice, has a slight cytotoxic activity in relation to mammalian cells, and stimulates tumor growth in mice, TNF production by human peripheral blood cells and proliferation of these cells.

The LPS macromolecule studied contains lipid A, the core oligosaccharide, and OPS. The hydrophilic portion of the lipid A macromolecule includes its characteristic components (glucosamine, ethanolamine, and phosphoethanolamine). The lipid A fatty acid profile is characteristic of the typical representatives of the genus *Pseudomonas* and *P. syringae* strains. The core contains all the components typical of this portion of the LPS macromolecule. The LPS O-chain is structured following the pattern common for all the *P. syringae* strains studied at present; the presence of rhamnan in the O-chain as its constituent and, thus, the regularity revealed earlier were confirmed with the use of a new strain. The established fact justifies the strain pv. *maculicola* IMV 381 assignment to the species *P. syringae*.

Close serological interrelationships between *P. syringae* pv. *maculicola* IMV 381 and the serogroup I strains [10] are more likely due to the presence of the common repeating unit with the structure (**2**) in the composition of their heterogeneous OPS.

The comparative studies of the chemical and biological properties of the virulent and avirulent dissociants showed that the degree of the LPS O-chain polymerism is one of the factors of *P. syringae* pv. *maculicola* IMV 381 virulence in model plant experiments and influences the in vitro activity of the LPS-mediated process of stimulation of TNF production by human peripheral blood cells and the proliferative response of these cells.

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