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EXPERIMENTAL ARTICLES

Composition, Structure, and Biological Properties of Lipopolysaccharides from Different Strains of *Pseudomonas syringae* pv. *atrofaciens*

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Abstract—The composition, structure, and certain biological properties of lipopolysaccharides (LPS) isolated from six strains of bacteria *Pseudomonas syringae* pv. *atrofaciens* pathogenic for grain-crops (wheat, rye) are presented. The LPS–protein complexes were isolated by a sparing procedure (extraction from microbial cells with a weak salt solution). They reacted with the homologous O sera and contained one to three antigenic determinants. Against the cells of warm-blooded animals (mice, humans) they exhibited the biological activity typical of endotoxins (stimulation of cytokine production, mitogenetic activity, etc.). The LCD of the biovar type strain was highly toxic to mice sensitized with D-galactosamine. The structural components of LPS macromolecules obtained by mild acidic degradation were characterized: lipid A, core oligosaccharide, and O-specific polysaccharide (OPS). Fatty acids 3-HO-$C_{10:0}$, $C_{12:0}$, 2-HO-$C_{12:0}$, 3-HO-$C_{12:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ were identified in lipid A of all the strains, as well as the components of the hydrophilic part: glucosamine (GlcN), ethanolamine (EtN), phosphate, and phosphoethanolamine (EtN-P). In the core LPS, glucose (Glc), rhamnose (Rha), L-glycero-D-manno-heptose (Hep), GlcN, galactosamine (GalN), 2-keto-3-deoxy-D-mannooctonoic acid (KDO), alanine (Ala), and phosphate were present. The O chain of all the strains consisted of repeated elements containing a linear chain of three to four L- (two strains) or D-Rha (four strains) residues supplemented with a single residue of 3-acetamido-3,6-dideoxy-D-galactose (D-Fuc*p*3Nac), N-acetyl-D-glucosamine (D-Glc*p*NAc), D-fucose (D-Fuc*f*), or D-Rha*p* (strain-dependent) as a side substituent. In different strains the substitution position for Rha residues in the repeated components of the major rhamnan chain was also different. One strain exhibited a unique type of O-chain heterogeneity. Immunochemical investigation of the LPS antigenic properties revealed the absence of close serological relations between the strains of one pathovar; this finding correlates with the differences in their OPS structure. Resemblance between the investigated strains and other *P. syringae* strains with similar LPS structures was revealed. The results of LPS analysis indicate the absence of correlation between the OPS structure and the pathovar affiliation of the strains.

Key words: Pseudomonas syringae pv. *atrofaciens*, lipopolysaccharide, O-specific polysaccharide, core oligosaccharide, lipid A, composition, structure, immunochemistry, biological activity.

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Lipopolysaccharide (LPS, O antigen, or endotoxin) is the main structural and functional component of gram-negative bacterial cell walls. These biopolymers determine to a large degree the character of the interactions of a microbial cell with its host macroorganism and with its environment as a whole. They participate in such biological processes as recognition, adhesion, antigen–antibody interaction, etc.

Application of chemotaxonomic criteria to bacterial classification is presently an urgent task. The composition and structure of LPS may be among these criteria, since this cell wall component of gram-negative bacteria determines their serological specificity. LPS is a complex macromolecule consisting of three structurally and functionally different components: lipid A, core polysaccharide, and O-specific polysaccharide (OPS). LPS biosynthesis therefore involves a number of various genetically determined reactions. Transformations in the genes encoding the LPS biosynthesis enzymes are required for any changes in the LPS structure to take place; thus, the LPS chemotype and therefore the serotype are relatively conservative characteristics with a possible taxonomic value.

Pseudomonas syringae is a group of phytopathogenic bacteria with similar physiological and biochemical characteristics. The members of this group are presently classified according to the plant from which they were originally isolated; such taxa were named

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pathovars [1]. This classification, however, is arbitrary, since the members of a pathovar are usually not strictly specific pathogens and are often able to infect a number of different host plants. The *P. syringae* pv. *atrofaciens* strains investigated were originally isolated from graincrops (rye, wheat, and oats) and are the major pathogens of these plants. The strains of this pathovar can, however, infect other plants; the degree of artificial infection varies within the range of 1–4 points [2].

Nine genomotypes have been identified within the *P. syringae* group by means of DNA–DNA hybridization and ribotyping of the type strains of each pathovar [3]. However, no other correlating characteristics that enable assignment of a strain to a particular genomotype have been revealed for most of them. Moreover, the genotyping was performed for a limited number of strains representing individual pathovars. No universally accepted classification of these organisms exists, in spite of numerous attempts of immune specificity-based classification [4–7].

Thus, the issues of taxonomy, classification, and host–pathogen specialization of *P. syringae* strains are presently not settled.

Since *P. syringae* strains can exist on plants in an epiphytic form [8] and occasionally cause epiphytotics, they are widespread in nature. Humans and animals repeatedly contact these microorganisms. We have determined that the LPS of the outer membrane of these bacteria are abundant, although but loosely bound. Understanding the degree of danger caused by these bacteria and, specifically, their endotoxins to warm-blooded organisms is therefore important.

A number of LPS have been recently characterized and the structures of the LPS O-chains established for *P. syringae* strains of different pathovars [9–11].

The present work provides a detailed comparative description of the properties of LPS isolated from *P. syringae* pv. *atrofaciens* belonging to the serological groups I, II, IV, V, and VI according to the classification [5] (isolation, purification, general composition, biological activity, and structural characteristics of the individual parts of the macromolecules). The goal of the work is to determine the applicability of the LPS characteristics as taxonomic criteria to assign a strain to a pathovar, as well as to determine the character of the biological activity of the LPS from phytopathogenic bacteria in respect to the cells of warm-blooded animals.

MATERIALS AND METHODS

The *Pseudomonas syringae* pv. *atrofaciens* strains IMV 4394 =ATCC 4394 (neotype), IMV 8281, IMV K1025, IMV 2846, IMV 948, and IMV 2399 were obtained from the collection of the Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. The strains were grown on potato agar at 26–28°C for 20–22 h.

The LPS were isolated from wet bacterial biomass by extraction with 0.85% NaCl solution in water and purified by repeated ultracentrifugation at 105000 *g* (three times for four hours).

For LPS degradation, the samples were treated with 1% CH_3COOH at 100°C for 1.5–3 h [12]. The precipitate of lipid A was centrifuged, washed with hot water, and after lyophilization was used as the lipid A fraction. The water-soluble fraction was separated chromatographically on a 65 × 1.6 cm column with the gel Sephadex G-50 (Pharmacia, Sweden) in 0.05 M pyridine–acetate buffer (pH 4.5). Elution was controlled by means of the phenol–sulfuric acid reaction. High-molecular fractions of the O-specific polysaccharide (OPS) and low-molecular fractions of the core oligosaccharide and lipid A were thus obtained for each strain.

The concentration of sugar in the LPS samples was determined by the phenol–sulfuric acid reaction; the concentration of proteins, by the Lowry method; of phosphorus, by the reaction with ammonium molybdate and ascorbic acid after mineralization; of KDO, by the reaction with thiobarbituric acid; and of heptoses, by the reaction with cysteine and sulfuric acid. The references to the publications describing these methods are cited in [13].

Lethal toxicity of the LPS preparations for 10–12-week mice of the BALB/c line sensitized with D-galactosamine and induction of the tumor necrosis factor (TNF-α) in human whole blood samples and in cultured mouse macrophages (by the cytotoxic effect on L929 cells and by immunoenzyme analysis) were determined as described in [14].

Cell lines L929 and P815 were obtained from the collection of cell cultures of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg, Russia) and maintained on the RPMI-1640 medium with sodium bicarbonate and L-glutamine in the concentrations recommended by the manufacturer (Sigma Chemical Co., United States), 40 μg/ml of gentamycin, and 5% of the calf fetal serum (CET) (Bio-Mark Inc., Lvov, Ukraine). Versene was used for cell passages. The RPMI-1640 medium without serum, supplemented with 25 mM of HEPES (Amimed, Switzerland), L-glutamine, and gentamycin, was used for all the experiments designated as HR.

In vitro determination of the cytotoxic and cytostatic activity of mice macrophages to P815 tumor cells was carried out as follows. Monolayers of peritoneal macrophages were incubated with or without LPS for 24 h, washed twice with hot HR medium, and incubated for 12 h with europium-labeled P815 cells (25000 cells per well). P815 cells were incubated in the medium with or without Triton X-100 (0.1%) in order to determine spontaneous and complete liberation of europium, respectively. After the incubation, 20 μl of supernatant from each well was transferred into the parallel wells of a 12-line, 96-well flat-bottomed plates

(Labsystems Oy, Finland); 200 μl of DELFIA solution (Wallac Oy, Finland) was added to each well, and europium fluorescence was measured with an ARCUS 1230 fluorometer (Wallac Oy) with a high temporal resolution. The ratio of lysed cells was calculated from the following equation:

$$\% \text{ Lysis} = (\text{EP} - \text{SR}/\text{TR} - \text{SR}) \times 100,$$

where ER is europium liberation in experimental wells with macrophages; SR, spontaneous europium liberation from the target cells incubated without LPS; and TR, total europium liberation from the detergent-lysed target cells.

After determination of the liberated europium, ^{3}H thymidine was added to the plate (0.5 μCi/well). In order to determine the proliferative activity of P815 cells incubated with the macrophages treated and untreated with LPS, the plates were then incubated for additional 18 h. The well content was then transferred on the filters of beta plates (Wallac Oy, Finland) with the help of an automatic harvester modulator (Skatron, Norway). Absorption of ^{3}H was determined on a beta plate counter (Wallac Oy, Finland). The inhibitory effect on growth of LPS-treated macrophages was expressed in percent form as a fraction of the inhibition of untreated macrophages.

Production of interferon (IFN2γ) and interleukin (IL-10) were determined with CLB commercial immunoenzyme kits (The Netherlands).

Rates of NO production (μM) in macrophage cultures were determined with the Griess reagent as described in [14].

Extraction of mouse splenocytes and determining their proliferation. In order to obtain splenocytes, the cell suspension was obtained from the spleen of 8–10-week mice of the BALB/c line. The splenocytes were washed twice with HR medium, counted, and resuspended in the growth medium (DMEM) with 4.5 g/l glucose and 110 μg/ml sodium pyruvate (D-7777; Sigma, United States). The medium was supplemented with 10% of bovine fetal serum, 40 μg/ml of gentamycin, and 50 μM of 2-mercaptoethanol. The cells were transferred (250000/well) to the wells of 96-well round-bottom plates for tissue cultures (Becton-Dickinson Labware, United States) with the medium or with the medium containing LPS in concentrations of 0.1, 1, and 10 μg/ml. The plates were incubated in a CO_2 incubator for 72 h. Eighteen hours before the end of incubation, ^{3}H thymidine (5 μCi/mmol) was added to a final concentration of 1 μCi/well. The content of the wells was then transferred on beta plate filters (Wallac Oy, Finland) with the help of an automatic harvester modulator (Skatron, Norway). Absorption of ^{3}H was determined on a beta plate counter (Wallac Oy, Finland). The results of proliferation response were presented in pulses/min. The average values of the responses of four cell samples were used for graph plotting.

Isolation of human mononuclear peripheral blood cells (MPBC) and analysis of proliferative response. MPBC from fresh, heparin-treated (15 U/ml), venous blood were centrifuged in Ficoll–Paque gradient (Pharmacia Biotech, Uppsala, Sweden), washed three times in a phosphate buffer, counted, and resuspended in complete IMDM medium (Sigma-Aldrich, United States) with 10% of human AB serum and 40 μg/ml of gentamycin at 106 cells/ml. Whole blood was diluted (1 : 5) with IMDM medium without serum. MPBC suspension or diluted whole blood (100 μl) was introduced into 96-well plates for tissue culture with 100 μl of LPS diluted in HR medium (Sigma–Aldrich, United States).

Determination of the LPS effect on T cells proliferation. In order to investigate the modulator effect of LPS of T cells proliferation, MPBC suspension or diluted whole blood (100 μl) was introduced into 96-well plates for tissue culture with 100 μl of LPS diluted in HR medium (Sigma–Aldrich, United States) supplemented with 100 ng/ml of anti-CD3 mitogenetic monoclonal antibodies (Mabs). The plates were incubated for four days in a CO_2 incubator. Eighteen hours before the end of incubation, ^{3}H thymidine (5 μCi/mmol) was added to a final concentration of 1 μCi/well. The content of the wells was then transferred on beta plate filters by means of an automatic harvester modulator (Skatron, Norway). Absorption of ^{3}H was determined on a beta plate counter. The results of MPBC proliferation response were presented in pulses/min after subtraction of spontaneous proliferation signals.

Determination of the LPS tolerance-stimulating activity. After stimulation with the LPS preparation (100 ng/ml) for 24 h, whole human blood cultures were washed three times with the warm HR medium and incubated for additional 18 h with *Escherichia coli* LPS O55:B5 (1 μg/ml) in HR medium with 5% of the homologous serum. The supernatants after the first and second incubation with LPS were collected and analyzed for TNF-α.

The preparation of immune sera and the reaction of immunoenzyme analysis (IEA) were carried out as described in [12].

Sugar analysis was performed by hydrolysis of the LPS, OPS, and the core oligosaccharide with 2 M trifluoroacetic acid (121°C, 120 min). After hydrolysate evaporation, the monosaccharides were converted into polyatomic alcohols by reduction with $NaBH_4$, acetylated with acetic anhydride in pyridine, and analyzed by gas–liquid chromatography (GLC) on a Hewlett–Packard 5880 chromatograph (United States) with a capillary column containing Ultra 2 as the stationary phase within the temperature gradient from 150 to 290°C (10°C/min). Gas–liquid chromatography–mass spectrometry was carried out on a Hewlett–Packard 5989 device (United States) equipped with a capillary column with HP-1 as the stationary phase [13].

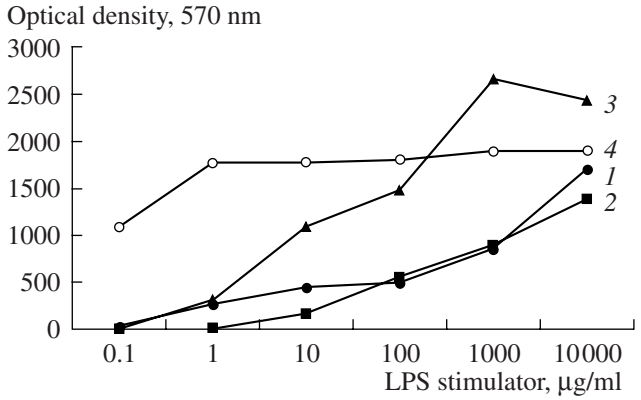


Fig. 1. Stimulation of TNF-α formation by the culture of whole human blood in the presence of LPS from strains IMV 4394 (*1*), IMV 2399 (*2*), *E. coli* O26:B6 (*3*), and *E. coli* O55:B5 (*4*).

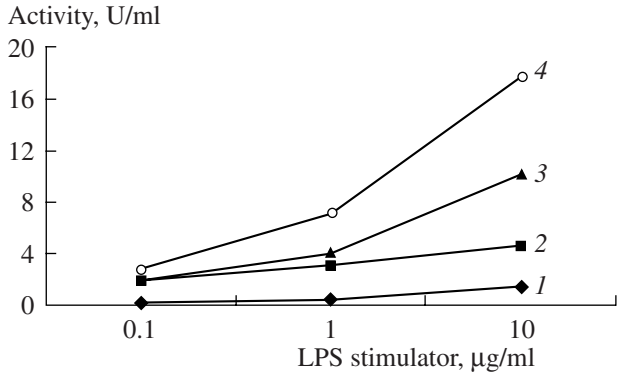


Fig. 2. Stimulation of TNF-α formation by mice peritoneal macrophages in the presence of LPS from strains IMV 4394 (*1*), IMV 2399 (*2*), *E. coli* O26:B6 (*3*), and *E. coli* O55:B5 (*4*).

Amino compounds (amino sugars, ethanolamine, phosphoethanolamine, and amino acids) were determined in hydrolysis products (1.0–2.0 mg of the sample, 0.5 ml 4 N HCl, 100°C, 18 h, sealed ampoules) with an AAA-339 analyzer (Czech Republic) with a 3.7×350 mm column with Ostion LG ANB cationite in the system of sodium citrate buffer stepwise gradients at 50–65°C [13].

The fatty acid composition of the LPS treated with methanol and chloroform in order to remove free phospholipids and of the lipid A fraction was determined according to the procedure described in [15].

The methods of structural analysis, including analysis of the monosaccharide composition, determination of the monosaccharide absolute configuration, OPS methylation, Smith degradation, gas–liquid chromatography–mass spectrometry, NMR spectroscopy, etc. have been described in [11, 13, 16].

RESULTS AND DISCUSSION

The LPS preparations obtained from *Pseudomonas syringae* pv. *atrofaciens* strains by the sparing method of washing with 0.85% NaCl contained 23.5–62.5% carbohydrates (determined by the phenol–sulfuric acid reaction) and a small amount of protein (0.7–3.0% as determined by the Lowry method). The preparation yield was 6.2–15.0%, depending on the strain. These results confirm our previous findings concerning the *P. syringae* group bacteria [9, 11, 13], namely, the high LPS content and their weak binding in the outer membrane.

The LPS preparations obtained from strains IMV 4394 and IMV 948 were toxic to D-galactosamine-sensitized mice of the BALB/c line. The LD_{50} values were 0.04 and 2.5 μg per mouse, respectively; the values for *E. coli* LPS O55:B5 and O26:B6 (Sigma, United States), which were used as positive controls, were 0.14 and 0.45 μg per mouse, respectively. Thus, The LPS from strain IMV 4394 had a more than three times higher toxicity than that from a toxigenic *E. coli* strain. Since strain IMV 4394 is among the major pathogens of grain cultures, the endotoxins of these bacteria liberated into the environment may be dangerous to animals and humans.

A number of other biological reactions in respect to the cells of warm-blooded animals (humans and mice) were determined for the LPS preparations from strains IMV 4394 and IMV 2399. Our results demonstrate that the biological activity of both experimental and control preparations depends both on the producer strain and the dose applied (Figs. 1–5, Tables 1–4).

The LPS preparations of the *P. syringae* strains investigated exhibited all the biological activities of the classical *E. coli* endotoxins used as a positive control. The activity was usually lower than in the case of *E. coli* LPS. However, the capacity of the LPS from *P. syringae* strains for the stimulation of mitogenetic

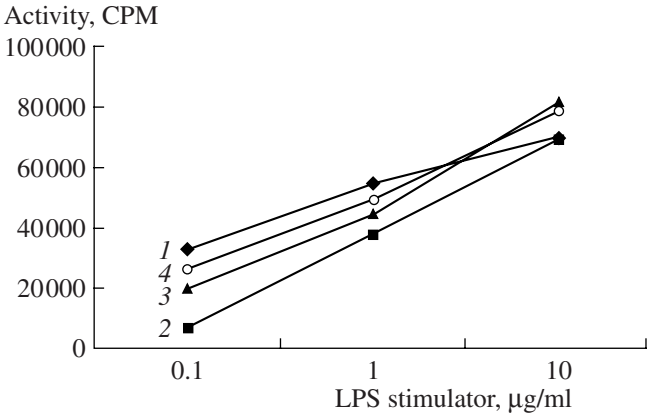


Fig. 3. Effect of LPS from strains IMV 4394 (*1*), IMV 2399 (*2*), *E. coli* O26:B6 (*3*), and *E. coli* O55:B5 (*4*) on the mitogenetic activity of mouse splenocytes.

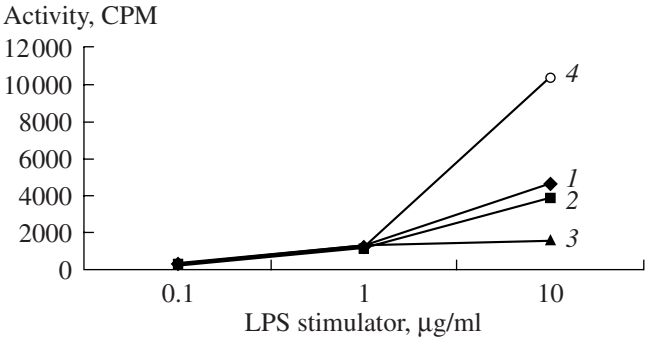


Fig. 4. Effect of LPS from strains IMV 4394 (*1*), IMV 2399 (*2*), *E. coli* O26:B6 (*3*), and *E. coli* O55:B5 (*4*) on the mitogenetic activity of the mononuclear cells of the culture of whole human peripheral blood.

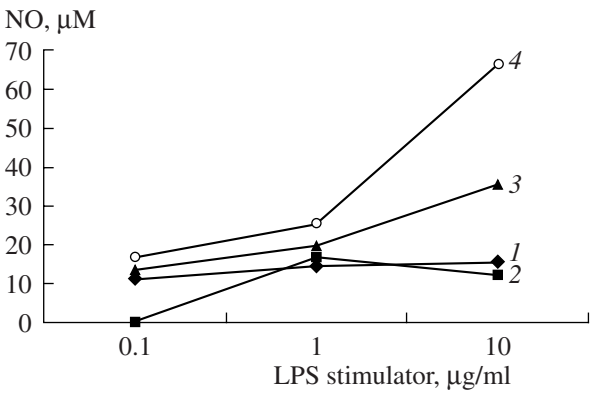


Fig. 5. Stimulation of NO formation by mouse peritoneal macrophages in the presence of LPS from strains IMV 4394 (*1*), IMV 2399 (*2*), *E. coli* O26:B6 (*3*), and *E. coli* O55:B5 (*4*).

activity (Figs. 4, 5) and tolerance (Tables 1, 2) was close to that of *E. coli* LPS. The preparation from strain IMV 4394 was more active in the stimulation of interferon-producing activity of human blood cultures than *E. coli* LPS (Table 1). The LPS from *P. syringae* strains influence the T component of the immune system; this is evident from suppression of T-cell-specific anti-CD3 proliferation by monoclonal antibodies (Table 3). Only the LPS preparation from strain IMV 4394 could lyse the cells of a very conservative tumor culture P815 (Table 4). The preparations of *E. coli* LPS exhibited only the cytostatic action.

Serological and immunochemical analysis has revealed [5, 17–20] that the LPS preparations isolated from different strains were active in reactions with homologous antisera obtained by immunizing the rabbits with whole microbial cells killed by heating. The titers of precipitation and passive hemagglutination reactions were approx. 1 : 12800–1 : 51120 and 1 : 640–1 : 5120, respectively. One to three precipitation bands were revealed by the Ouchterlony precipitation reaction. The O antigenic schemes of the investigated strains, including up to three group-specific O antigenic factors, were determined by reactions of agglutination and agar precipitation with whole and cross-adsorbed sera. Cross group agglutination reactions, double diffusion in agar, and hemagglutination revealed only a weak relationship between the *P. syringae* pv. *atrofaciens* strains from different serogroups. According to the serological data, strain IMV 8281 occupied an isolated position within the pathovar.

After methanolysis of the LPS preparations from all the strains investigated, The GLC analysis revealed methyl ethers of the same fatty acids, including the 2-hydroxy- and 3-hydroxyalkane acids typical of the pseudomonad lipid A [15]. The components identified by GLC as acetates of polyatomic alcohols, ion exchange chromatography on an amino acid analyzer, and colorimetric analysis are listed in Table 5.

Table 1. Biological activity of the LPS from the investigated *P. syringae* pv. *atrofaciens* strains in the cultures of whole human blood

LPS from strain	LPS treatment					
	First: 0.1 μg/ml				Second: 1 μg/ml	
	TNF-α, pg/ml		IL-10	IFN-γ	TNF-α	Tolerance*, %
	Total	Bioactive	pg/ml			
Spontaneous	973	580	12	1679	624	0.0
E. coli O55:B5	4380	3310	75	931	104	83.3
E. coli O26:B6	4049	2805	103	1231	80	87.2
IMV 4394	1023	755	34	1674	80	87.2
IMV 2399	1691	1140	32	524	88	85.9

Notes: * % of inhibition by different *P. syringae* LPS (dose, 0.1 μg/ml) of TNF-α formation by whole human blood cells in response to stimulation with *E. coli* O55:B5 LPS (dose, 1 μg/ml); TNF-α, tumor necrosis factor; IL-10, interleukin; IFN-γ, interferon.

Table 2. Concurrent effect of the LPS from *P. syringae* pv. *atrofaciens* strains and *E. coli* O55:B5 on TNF-α (pg/ml) production in the culture of native human blood

LPS from strains	*E. coli* O55:B5 LPS	LPS concentration, ng/ml					
		0	1	10	100	1000	10000
E. coli O55:B5	Without *E. coli* LPS	0	1546	1883	1918	2249	2255
	1 ng/ml *E. coli* LPS	2348	2585	2443	2543	2378	2420
	Control, %	100.0	**129.6**	**120.4**	**145.3**	**130.3**	**177.4**
E. coli O26:B6	Without *E. coli* LPS	0	0	1124	1837	1822	1393
	1 ng/ml *E. coli* LPS	2348	2377	2537	2487	2498	1960
	Control, %	100.0	**101.2**	**115.4**	**127.2**	**128.5**	59.4
IMV 4394	Without *E. coli* LPS	0	0	767	848	989	1046
	1 ng/ml *E. coli* LPS	3553	3136	2836	1933	1286	1253
	Control, %	100.0	88.3	74.3	40.1	11.6	8.3
IMV 2399	Without *E. coli* LPS	0	0	0	770	1103	1048
	1 ng/ml *E. coli* LPS	3035	2774	2785	2854	2062	1891
	Control, %	100.0	91.4	91.8	92.0	49.6	42.4

Table 3. Effect of the LPS from *P. syringae* pv. *atrofaciens* strains on T cell proliferation (CPM)

LPS from strains:	Anti-CD3 monoclonal antibodies	LPS concentration, ng/ml				
		0	1	10	100	1000
E. coli O55:B5	Without antibodies	782	939	590	447	482
	100 hg/ml	10275	11552	9921	8222	8092
	Control, %	100.0	111.8	98.3	81.9	80.2
E. coli O26:B6	Without antibodies	782	514	637	733	461
	100 hg/ml	10275	12070	10120	10717	10581
	Control, %	100.0	121.7	99.9	105.2	106.6
IMV 4394	Without antibodies	649	360	536	612	596
	100 hg/ml	19140	18429	19730	18927	10445
	Control, %	100.0	97.7	103.8	99.0	53.3
IMV 2399	Without antibodies	661	472	457	686	787
	100 hg/ml	18117	19196	18418	16112	13126
	Control, %	100.0	107.3	102.9	88.4	70.7

Table 4. Effect of LPS-treated macrophages on the P815 cell culture

LPS from strain	Lysis, %			Cytostatic activity, %		
	LPS concentration, μg/ml			LPS concentration, μg/ml		
	0.1	1	10	0.1	1	10
E. coli O55:B5	0	0	0	1.6	12.0	18.0
E. coli O26:B6	0	0	0	1.3	9.8	15.6
P. syringae pv. *atrofaciens* IMV 4394	0	9.6	**13.0**	2.4	9.3	10.3
P. syringae pv. *atrofaciens* IMV 2399	0	0	0	0	10.1	0

Apart from hydroxylated fatty acids, such characteristic LPS components as KDO and heptose were revealed in all the preparations. Similar to the previously studied *P. syringae* strains [9, 10], rhamnose was the major neutral sugar of the LPS of all strains. However, the monosaccharide composition of the preparations was not identical. The specific components revealed include 3-aminofucose (strain IMV 8281) and fucose (strain IMV 2399). Compared to the LPS of other strains, those of IMV 4394 and IMV 2846 had higher glucosamine content; this sugar was therefore possibly present in the O chain.

Mild acidic degradation with the subsequent chromatography on a Sephadex G-50 gel column revealed the fractions of OPS and core oligosaccharide; the lipid A fraction was obtained as a water-insoluble precipitate. The yield of the individual fractions is presented in Table 6.

The curves of polysaccharide elution from Sephadex columns (the elution curve for the PS of strain 8281 is presented on Fig. 6) and the qualitative ratio of the fractions (Table 6) indicate the predominance of the S form LPS molecules in the original preparations from all the strains. This distribution has been reported for other members of this bacterial group [9]. The macromolecular organization of the LPS of these strains is therefore typical of this group of glycopolymers. The LPS of strain IMV 4394 differs from the other preparations investigated. It contains a comparatively higher amount of the core fraction and less of lipid A. This characteristic structure of the LPS macromolecule of this strain may probably explain its higher toxicity and the differences in its other biological features (Figs. 1–5, Tables 1–4).

In the lipid A fraction of all the LPS investigated, chromato–mass spectrometry revealed the characteristic ions of the following fatty acids: 3-HO-$C_{10:0}$, $C_{12:0}$, 2-HO-$C_{12:0}$, 3-HO-$C_{12:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$. The strains differ in the ratio of these acids (Table 7).

Our findings concerning the fatty acid composition of *P. syringae* pv. *atrofaciens* are close to the results obtained previously for strains of *P. syringae* [9, 11, 15] and *P. fluorescens* [12, 21]. Apart from the 3-HO-$C_{10:0}$, $C_{12:0}$, 2-HO-$C_{12:0}$, and 3-OH-$C_{12:0}$ fatty acids, which have been established as the lipid A components of pseudomonads [11, 13, 15], all the investigated preparations of lipid A contain such nonhydroxylated fatty acids with a longer carbon chain as $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$. Their presence does not depend on the method of LPS isolation from the cells and its purification (including preliminary treatment with a chloroform–methanol mixture for phospholipid removal and repeated washing with hot water). These acids are not removed by dephosphorylation with 48% hydrofluoric acid and are present in the preparations of both cellular and extracellular LPS (the latter is obtained from the mixture of secondary metabolites which the living microbial cells excrete into their environment) [12].

Table 5. Overall composition of LPS from *P. syringae* pv. *atrofaciens* strains

Component	LPS from strains:					
	8281	K1025	4394	2846	948	2399
% of total (peak area, GLC)						
Rha	**91.5**	**73.7**	**95.6**	**96.1**	**93.3**	**68.7**
Glc	8.5	11.7	1.4	0.5	6.2	3.5
Fuc	–	–	–	–	–	**17.0**
Xyl	–	–	–	trace	–	5.8
Gal	–	3.2	2.0	2.0	0.4	–
Man	–	5.6	0.3	0.8	trace	4.9
X	2.1	5.6	0.5	0.5	trace	trace
% of dry weight						
GlcN	+	1.8	**7.8**	**9.0**	3.6	2.0
GalN	+	1.0	0.6	0.8	1.8	0.9
Fuc3N	4.8	–	–	–	–	–
KDO	+	0.7	0.3	trace	0.8	0.7
Hep	2.1	trace	trace	0.1	1.6	trace
Ala	+	2.4	1.4	1.6	1.7	2.0

Notes: X, peak at R_{Rha} = 1.13–1.15; (–), absence of feature; (+), presence of feature; Rha, rhamnose; Glc, glucose; Fuc, fucose; Xyl, xylose; Gal, galactose; Man, mannose; GlcN, glucosamine; GalN, galactosamine; Fuc3N, 3-aminofucose; KDO, 2-keto-3-deoxyoctulonic acid; Hep, heptose; Ala, alanine.

Table 6. The fractions representing the structural components of *P. syringae* pv. *atrofaciens* strains LPS

Strain	Components of the LPS molecule, wt % of the original dry preparation			
	OPS	Core oligosaccharide		Lipid A
	I fraction*	II fraction*	III fraction*	
IMV 8281	43.3	11.0	8.7	37.9
IMV K1025	29.0	7.2	3.6	60.0
IMV 4394	39.5	13.8	10.7	35.9
IMV 2846	38.3	8.3	6.7	46.7
IMV 948	34.1	10.2	2.3	53.4
IMV 2399	35.6	8.0	4.0	53.0

Notes: * Polysaccharide fractions similar to those presented as an example for the LPS from *P. syringae* pv. *atrofaciens* strain IMV 8281 (Fig. 6).

Other authors have reported $C_{16:0}$ and $C_{18:0}$ fatty acids in the LPS from *P. syringae* pv. *phaseolicola* [22] and *P. aeruginosa* [23].

Glucosamine and phosphoethanolamine, which were detected in the hydrophilic part of lipid A from all the strains under investigation (Table 8), are the typical

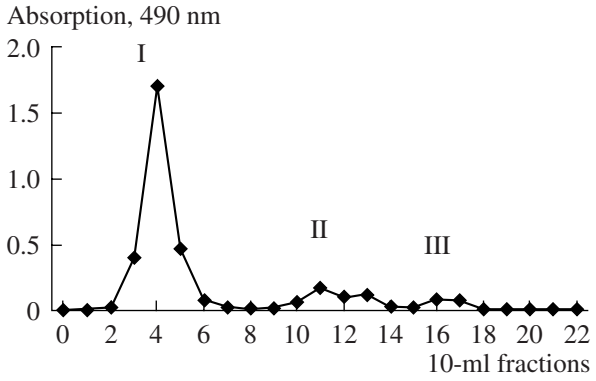


Fig. 6. Elution curve of *P. syringae* pv. *atrofaciens* IMV 8281 PS (Sephadex G-50). I, peak corresponding to the fraction of O-specific polysaccharide; II, III, peaks corresponding to the fractions of core oligosaccharide.

components of this part of LPS macromolecules. The ratio of the components revealed does not differ significantly in different strains. Only the lipid A from strain 4394 contains more glucosamine than the preparations obtained from other strains. The lipids A of all the strains contained phosphate and did not contain neutral sugars (according to the GLC results). Thus, lipid A of all the strains investigated contains all the characteristic components; it, however, has a low degree of phosphorylation (with the exception of strain 8281).

Rhamnose and glucose were revealed as the major components of the core oligosaccharide fraction (Table 9) by means of GLC as acetates of polyatomic alcohols). The presence of L-glycero-D-manno-heptose, KDO, and phosphate was revealed by colorimetric analysis. The neutral sugars found (rhamnose, glucose, and heptose) are the typical components of the LPS core in pseudomonads [9, 13, 21]. One *P. fluorescens* strain is exceptional in containing no rhamnose [23]; this sugar is therefore not a necessary component of the LPS core in pseudomonads.

After hydrolysis of the OPS fractions with 2 M CF_3COOH, the monosaccharides listed in Table 10 were revealed by GLC as acetates of polyatomic alcohols, by ion exchange chromatography on an amino acid analyzer, and by the GLC analysis of acetylated glycosides with (–)-2-octanole.

Comparative analysis of these data demonstrates the presence of such sugars as D-rhamnose, D-fucose, and 3-acetamido-3-deoxy-D-fucose in the OPS of the strains of this pathovar; these sugars, seldom occurring in nature, are the typical components of *P. syringae* LPS [9, 10]. The LPS of different strains differ in the monosaccharide composition of the O chain. For the six strains investigated, five chemotypes of the O chain were revealed; these finding correlates with the five serogroups comprising these strains [5, 19]. Strains IMV 4394 and IBM 2846 have an identical OPS monosaccharide composition and belong to the same serogroup IV [5]. The complete structural analysis of the OPS fraction of the LPS of the investigated strains of *P. syringae* pv. *atrofaciens* was performed by means of NMR spectroscopy (including its various modifications), GLC–mass spectrometry, methylation, Smith degradation, partial acid hydrolysis, and other techniques.

NMR spectroscopy revealed that the OPS of strain IMV 8281 LPS is not strictly regular. The presence of the signals of different intensity in ^{13}C and ^{1}H NMR spectra (Fig. 7) confirmed this fact. The structural analysis confirmed that the OPS of this strain consisted of three types of repeated oligosaccharide components with structures 1, 2, and 3 (Table 11); alternatively, the LPS pool of this strain may contain three types of molecules with regular polysaccharide O chains built of repeated 1, 2, and 3 components, respectively. The rhamnose and Fuc3NAc ratio determined by GLC and NMR spectroscopy is 3.4 : 1; the ratio between the partially methylated rhamnose derivatives (2-substituted, 3-substituted, and 2,3-disubstituted) and the terminal Fuc3N is 0.65 : 1.7 : 1: 0.3, respectively; and the comparative analysis of integral intensities of the signals of

Table 7. Quantitative ratios of fatty acids in lipids A from different *P. syringae* pv. *atrofaciens* strains

Fatty acid	Lipids A from strains:					
	IMV 8281	IMV K1025	IMV 4394	IMV 2846	IMV 948	IMV 2399
	% of total (peak area, GLC)					
3-HO-$C_{10:0}$	6.8	1.5	5.9	4.8	2.4	3.9
2-HO-$C_{12:0}$	13.7	5.9	10.9	8.7	8.5	9.1
3-HO-$C_{12:0}$	17.2	5.2	7.5	8.7	8.8	8.1
$C_{12:0}$	37.2	3.9	15.1	17.6	10.1	14.8
$C_{16:1}$	0.6	27.6	16.4	13.5	18.0	17.9
$C_{16:0}$	21.1	33.4	21.1	20.0	31.1	28.8
$C_{18:1}$	0.6	17.1	19.6	19.3	4.8	10.1
$C_{18:0}$	2.7	2.6	3.1	2.0	16.8	2.3

anomer protons in ^{1}H NMR spectra of the polysaccharide after Smith degradation and of ^{13}C and ^{1}H NMR spectra of original polysaccharides indicate a ratio between repeated components 1, 2, and 3 of ~6 : 2.3 : 1. The structures of repeated components 1, 2, and 3 were determined by various modifications of NMR spectroscopy and by Smith degradation (Table 11). The structure of the OPS from *P. syringae* pv. *atrofaciens* strain IMV 8281 was described in detail in [16].

The data presented demonstrate that the heterogeneity of the OPS from strain IMV 8281 is related to the variation in the number of rhamnose residues in the repeated component and to the substitution position in one of these residues. The heterogeneity of the second type have been previously determined for the O polysaccharides of some strains of *P. syringae, Burkholderia cepacia*, and *Ralstonia solanacearus* [10]. However, according to our knowledge, the heterogeneity of the first type has not been described for microbial lipopolysaccharides.

The spectra of ^{1}H and ^{13}C NMR of the OPS from *P. syringae* pv. *atrofaciens* strain IMV 948 also contained the signals of different intensity (Fig. 8); the main series was easily discernable, which belonged to a repeated pentasaccharide component with 4 L-Rha residues and one of D-GlcNAc. The complete structure of the repeated OPC component of the *P. syringae* pv. *atrofaciens* IMV 948 LPS was determined by the methods of methylation and GLC–mass spectrometry of the partially methylated acetates of polyatomic alcohols and by various modifications of NMR spectroscopy (Table 11, structure 6).

Among the minor monosaccharide residues of the OPS, only the ^{1}H NMR signal of one Rha residue was completely associated (H1–H6 at 5.06, 4.15, 3.92, 3.60, 3.88, and 1.33 ppm, respectively). Considering the position of C1 and C3 signals at 103.0 and 79.3 ppm, respectively, as determined from the two-dimensional HMQC spectrum, this residue is substituted in position 3 and is not substituted in position 2. The signals of the other residues of the minor series were not associated, since their signals coincided with those of the major series; the exact structure of the minor repetitive component was therefore not determined. According to the relative integral signal intensity in ^{1}H NMR spectra of the OPS, the ratio of the major and minor repetitive components is ~3.5 : 1.

In the case of *P. syringae* branched OPS with four L-Rha residues in the repeated component of the main chain, heterogeneity is known [9, 10] that is related to the presence of two repeated tetrasaccharide components of the main chain; the major one among these contains two Rha residues substituted in positions 2 and 3, while the minor one contains three Rha residues substituted in position 3 and one substituted in position 2. Since the structure of the OPC main repeated component in *P. syringae* pv. *atrofaciens* IMV 948 follows this pattern, structure 7 was tentatively suggested for the

Table 8. Components of the hydrophilic part of lipids A of the investigated strains, % of dry weight

Component	Strains					
	8281	K-1025	4394	2846	948	2399
GlcN	3.9	1.8	6.2	4.2	5.9	1.7
EtN-P	1.6	0.5	1.8	1.1	2.3	0.5
EtN	0.7	0.2	0.8	1.3	0.7	0.2
X	1.5	0.8	1.5	1.2	1.4	0.8
P	3.4	1.2	1.2	1.9	1.4	1.7

Notes: X, unidentified component with the relative retention time on an amino acid adalyzer T_{NH_3} 0.63; EtN, ethanolamine; EtN-P, ethanolamine phosphate; P, phosphorus.

Table 9. Major components of the core oligosaccharide of *P. syringae* pv. *atrofaciens* strains

Component	Strains					
	8281	K-1025	4394	2846	948	2399
% of total (peak area, GLC)						
L-Rha	26.8	41.3	40.7	27.7	26.6	32.2
D-Glc	73.1	58.6	59.2	72.2	71.3	63.1
% of dry weight						
LD-Hep	4.2	+	+	+	4.4	6.6
KDO	+	+	+	4.6	2.6	2.7
D-GlcN	1.6	5.4	2.9	6.0	4.6	2.8
D-GalN	1.7	3.1	3.0	5.1	3.4	2.0
Ala	2.3	2.0	2.5	2.5	3.7	1.4
P	2.7	4.5	ÒÎ	4.8	2.8	2.6

Note: Designations as in Tables 5 and 8.

Table 10. Sugar composition of the OPS fraction of the LPS from *P. syringae* pv. *atrofaciens* strains

LPS from strain	Sugar residues				
	L-Rha	D-Rha	D-Fuc	D-GlcNAc	D-Fuc3NAc
IMV 8281	+	–	–	–	+
IMV K1025	–	+	–	–	–
IMV 4394	–	+	–	+	–
IMV 2846	–	+	–	+	–
IMV 948	+	–	–	+	–
IMV 2399	–	+	+	–	–

Notes: (+), presence of feature; (–), absence of feature; L- and D-Rha, L- and D-rhamnose; D-Fuc, D-fucose; D-Fuc3Nac, 3-acetamido-3-deoxy-D-fucose; D-GlcNAc, N-acetyl-D-glucosamine.

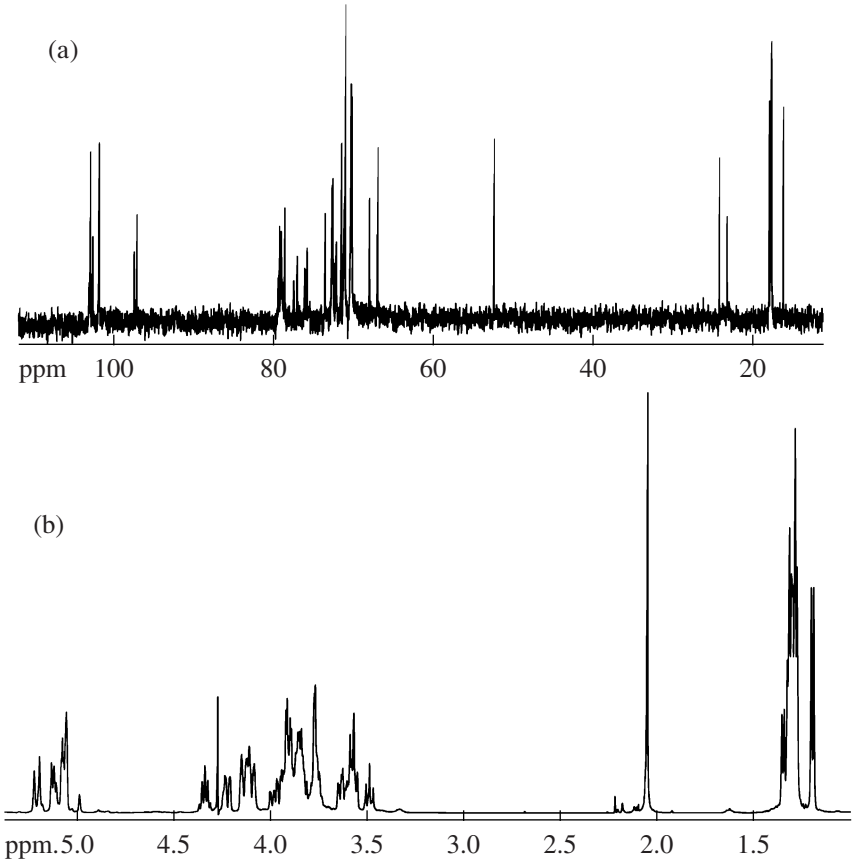


Fig. 7. ^{13}C- (a) and ^{1}H (b) spectra of *P. syringae* pv. *atrofaciens* IMV 8281 OPS.

minor repetitive components (Table 11). It is confirmed by the identification of a signal of the Rha residue substituted in position 3 in the minor series of the ^{1}H NMR spectrum; this signal is not present in the major series of the spectrum. Analysis of the OPS of the LPS of this strain has been presented in detail in [11].

Comparison of the results of the structural analysis (Table 11) demonstrates that the OPS of the investigated strains of *P. syringae* pv. *atrofaciens* differ in the structure of the repetitive component of the LPS O chain. The OPS heterogeneity of these strains resulted in detection of eight structural types of O chains for five chemotypes. The results of the structural analysis correlate well with the distribution of the strains within five serogroups. Strains IMV 4394 and IMV 2846 both belong to serogroup IV and have an OPS of identical composition. In spite of their differences, the OPS of all the strains investigated have a high degree of structural resemblance. All the repetitive components contain a linear rhamnan as their basis. The structure of the rhamnans of the major repetitive components is identical within the strain groups: IMV K1025, IMV 4394, IMV 2846, IMV 2399, and IMV 8281, IMV 948. In the OPS of strains IMV 4394, IMV 2846, and IMV 948, the rhamnans are substituted by the same lateral element, β-D-GlcNAc.

Comparison with the previously described structures revealed (Table 12) the identity of structure 3 of the heterogeneous OPS from strain IMV 8281 (Table 11) and the OPS structure of *P. syringae* pv. *tabaci* IMV 223. This is also one of the two structures of the heterogeneous OPS from the type strain *P. fluorescens* IMV 4125 [21]. Structural similarities exist also between the OPS from strain IMV 2399 and from strains *P. syringae* pv. *syringae* IMV 218 and IMV P-55 (an identical lateral substitute -α-D-Fuc*f* and a section of the main rhamnan chain). OPS from strains IMV 4394, IMV 2846, and IMV 948 contain the same lateral substitute of the rhamnan chain as those from *P. syringae* pv. *tabaci* IMV 225 (β-D-GlcNAc).

The results of structural analysis correlate with those of immunochemical investigation. The IEA reaction of the LPS from different *P. syringae* pv. *atrofaciens* strains with polyvalent antisera to microbial cells killed by heating (Table 13) revealed close serological similarity between the strains belonging to the same serogroup and sharing the OPS structure. In spite of the above characteristics of OPS structural similarity (Table 11), the serological relationship between the *P. syringae* pv. *atrofaciens* strains from different serogroups are practically absent (Table 13). The reaction between O antiserum to microbial cells with the homol-

Table 11. OPS composition of the LPS from *P. syringae* pv. *atrofaciens* strains

Strain, reference	Serogroup	Structure of the repetitive O chain component
IMV 8281 [16]	I	α-D-Fuc*p*3NAc 1 ↓ 3 →2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ (*1*) (65%) α-D-Fuc*p*3NAc 1 ↓ 3 →3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ (*2*) (25%) α-D-Fuc*p*3NAc 1 ↓ 3 →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→ (*3*) (10%)
IMV K1025 [26]	II	α-D-Rha 1 ↓ 3 →2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ (*4*)
IMV 4394, IMV 2846 [24]	IV	~10–100% β-D-GlcNAc 1 ↓ 4 →2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ (*5*)
IMV 948 [11]	V	β-D-GlcNAc 1 ↓ 2 →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*) (~80%) →2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ (*7*) (~20%)
IMV 2399 [25]	VI	α-D-Fuc 1 ↓ 4 →2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ (*8*)

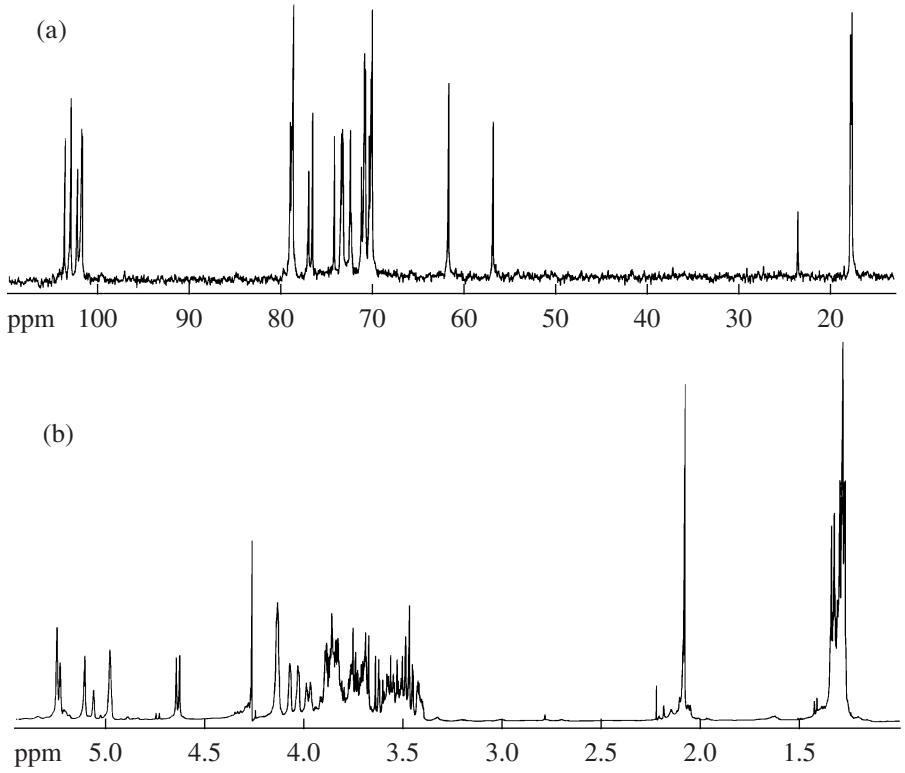


Fig. 8. ^{13}C- (a) and ^{1}H (b) spectra of *P. syringae* pv. *atrofaciens* IMV 948 OPS.

ogous LPS was completely inhibited only by the homologous LPS and the LPS from the strain belonging to the same serogroup, independently on its pathovar identification. The weak serological relationship (between strains IMV 4394, IMV 2846, IMV L-25, and IMV 948) was probably due to the antigenic determinants located in the core lipid part of the LPS molecule, rather than in its O-specific polysaccharide chain. This suggestion is supported by the results on the serological activity of the preparations of individual fractions corresponding to different structural parts of the LPS macromolecules (Table 14).

These data demonstrate (Table 14) that the OPS fractions exhibited high serological activity in the IEA reaction with the O antiserum to microbial cells of various *P. syringae* pv. *atrofaciens* strains killed by heating and to homologous LPS; this activity, however, was usually lower than that of LPS. The core oligosaccharide exhibited lower serological activity in a homologous test system; in the case of the LPS from strain IMV K1025, the core oligosaccharide was serologically inert. The main immunodominant fragments of the LPS are therefore localized on the OPS. This finding is supported by the serological isolation of strain IMV K1025 among the other strains of the pathovar (see Table 13).

Weak (13–34%) cross reactions with the LPS of other strains and pathovars were detected (Table 13); they may be due to the identity of O chains (structure 3, strains 8281 and 223, Tables 11, 13) or to the determinants located in the core lipid part of the LPS macromolecule.

The absence of serological relations between the strains with identical structures of the major rhamnan chain of the repetitive OPS component (e. g., strains IMV K1025, IMV 4394, and IMV 2846) indicates its immunological inertness in the presence of branching lateral substitutes.

Thus, O antigens were isolated from the biomass of six *P. syringae* pv. *atrofaciens* strains. The isolation and characterization of these antigens confirmed all the characteristics of their organization in bacteria of the *P. syringae* group that have been previously determined by us and by other authors [9, 10, 13]. The content of O antigen was high; it was weakly bound to the outer membrane (easily washed with the salt solution); the LPS preparations contained mainly S forms of the molecules; the lipid A was built according to the known concepts concerning the organization of these biopolymers; it contains the fatty acids commonly present in typical pseudomonads. *P. syringae* strains differ from the other pseudomonads investigated in the lower degree of lipid A phosphorylation. This feature is known to be very important for the toxic effect of endotoxins [13].

In the structure of the core oligosaccharide, some differences between the strains were revealed. Similar to lipid A, the differences in the core composition did

Table 12. OPS composition [10] of the LPS from the strains of other *P. syringae* pathovars and serogroups with a similar O chain structure

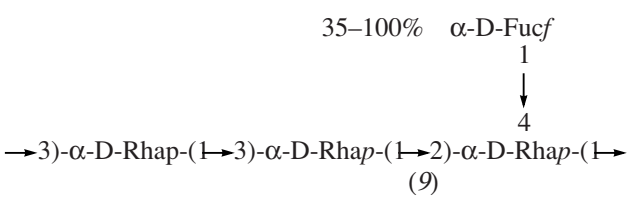
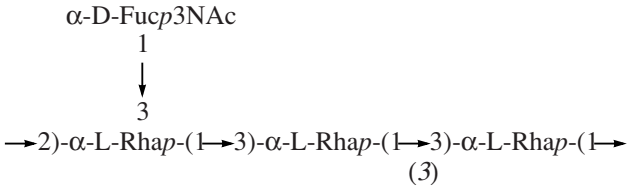
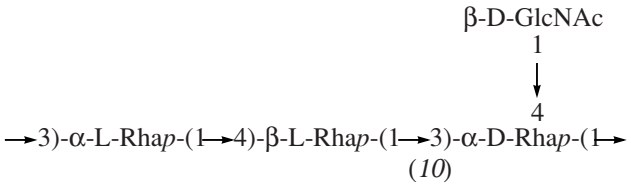
Pathovar, strain	Serogroup [5]	Structure of the repetitive LPS O chain component
pv. *syringae* IMV 218 pv. *syringae* IMVR-55	II III	 (*9*)
pv. *tabaci* IMV 223	VII	 (*3*)
pv. *tabaci* IMV 225	VIII	 (*10*)

Table 13. Serological relations between *P. syringae* pv. *atrofaciens* strains and other strains of the *P. syringae group**

P. syringae pv. *atrofaciens* strains	Serogroup [5, 19]	LPS inhibitor producing strain, serogroup [5, 19]		Inhibition**, %
IMV 8281	I	Strains of serogroup	I	100
		pv. *tabaci* 223	VII	34
IMV K1025	II	Strains of serogroup	II	42–100
IMV 4394, IMV 2846	IV	Strains of serogroup	IV	90–100
		pv. *atrofaciens* 948	V	5
		pv. *tabaci* 225	VIII	13
IMV 948	V	Strain 948	V	100
		pv. *atrofaciens* 4394	IV	5
		pv. *glycinea* L-25	IV	20
		pv. *tabaci* 225	VIII	28
IMV 2399	VI	Strains of serogroup	VI	100
		pv. *syringae* P-55	III	18
		pv. *syringae* 218	II	24

Notes: * Relations determined based on the interaction between O antisera to microbial cells killed by heating and LPS preparations in the IEA reaction.

** Maximal retardation at the LPS inhibitor concentration up to 200 μg/ml.

Table 14. Serological activity* of the different components of the LPS macromolecule

O serum + LPS of strain:	Maximum inhibition (%) by the preparations of:			
	LPS	OPS	Core	Lipid A
IMV 8281	100	98	55	96
IMV K1025	100	62	0	ND
IMV 4394	100	63	13	54
IMV 948	100	100	32	93

Notes: ND, not determined.
* IEA inhibition in the homologous system: O serum to microbial cells killed by heating–anti-LPS of a homologous strain.

not correlate with the strain pathogen–host differentiation and serogrouping. The main feature of the LPS O chain organization known for other *P. syringae* strains (the presence of a linear D- or L-rhamnan as its base) was confirmed. This characteristic can be used as one of the chemotaxonomic criteria for classification of *P. syringae* strains. The LPS from *P. syringae* pv. *atrofaciens* IMV 4394 exhibited high toxicity for warm–blooded organisms. The relation was revealed between the pathogen–host differentiation at the level of O chain structure; the strains of serogroups I and IV which differed in their OPS structure have been isolated from infected plants of rye and wheat, respectively [20]. The LPS O chain structure correlates with the serogrouping of the strains.

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