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EXPERIMENTAL ARTICLES

Pseudomonas syringae Lipopolysaccharides: Immunochemical Characteristics and Structure as a Basis for Strain Classification

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Abstract—Lipopolysaccharide (LPS) preparations of 34 *Pseudomonas syringae* strains of 19 pathovars were prepared by saline extraction from wet cells and purified by repeated ultracentrifugation. The preparations reacted with homologous O-antisera, obtained by rabbit immunization with heat-killed bacterial cells. Through inhibition of homologous reactions between LPS preparations of heterologous strains (enzyme immunoassay, EIA), it was established for the first time that high serological affinity between strains is observed only if their LPS contains O-specific polysaccharide chains (OPS) comprised of completely identical rather than partially similar units. The central linear part of the OPS was found to be serologically inert when shielded with side groups. Data on immunochemical characteristics of the LPS and OPS structure are analyzed in relation to the design of *P. syringae* classification scheme.

Key words: Pseudomonas syringae, classification, lipopolysaccharide, O-specific polysaccharide, structure, immunochemistry.

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Lipopolysaccharide (LPS) is a unique structural component of cell walls of gram-negative bacteria and the major antigen of the bacterial outer membrane. Residing immediately on the surface of the bacterial cell, LPS plays a crucial role in the processes of interaction with other microorganisms, bacteriophages, host organisms, and the environment in general. Since the individual structural components of the LPS macromolecule (see figure) differ in structure and functions and are of varying degree of conservatism, LPS structure and composition are among the acknowledged chemotaxonomic criteria used to evaluate phylogenic affinity between bacteria and trace their evolution. The fine structure of O-antigens determines the immunospecificity of bacterial cells (serotype and serogroup) and, presumably, the specificity of recognition and interaction with the host plant (pathovar). Therefore, LPS investigation is important for various aspects of modern biology and microbiology.

For several decades, most studies concentrated on the LPS of human and animal pathogens. However, lately, LPS of conditionally pathogenic and phytopathogenic bacteria have been attracting growing attention. *Pseudomonas syringae*, which attacks most cultured plant species [1], is among such microorganisms. Together with their great abundance and the well-known bacterial polybiotrophic ability, this implies possible adverse effects of the bacteria, particularly via their metabolites, on both humans and animals.

The taxonomic status of *Pseudomonas syringae* has been being discussed for years, yet it has not been established so far. Depending on the ability of *P. syringae* strains to affect a particular plant (or plants), they are divided into more than 50 pathovars [1], which are not yet classified. Attempts were made to classify *P. syringae* strains basing on the results of ribotyping and DNA–DNA hybridization of the reference strains of each pathovar [2], as well as on their immunospecificity [3–5]. To date, none of the classification schemes has been universally acknowledged. Therefore, the search for new, more conserved characteristics to assign strains to various pathovars, genomotypes, and serotypes and create a universal classification approach is always a topical issue.

Structural analysis of OPS comprising the LPS of 55 *P. syringae* strains representing all the known serotypes of these bacteria performed by us and other researchers [5–8] revealed 32 chemotypes of repeating units (Table 1). All of the repetitive units identified are generally based on the presence of a linear L-, D-, or L/D-rhamnan in the chain. Rhamnan-based OPSs consist of tri- or tetrasaccharide repeating units in the main chain and of side groups represented by D-rhamnose (D-Rha), D-fucose (D-Fuc), *N*-acetyl-D-glucosamine (D-GlcNAc), *N*-acetyl-3-amino-D-fucose (D-Fuc3NAc), and O-methyl (O-Me) group.

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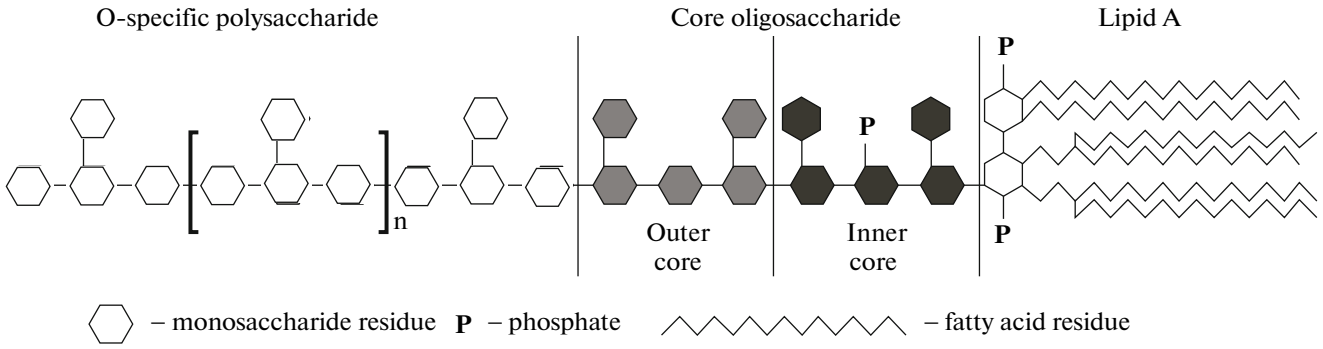

Schematic representation of the structure of a lipopolysaccharide molecule in gram-negative bacteria.

The aim of the present work was to study the serological affinity between *P. syringae* strains by enzyme immunoassay (EIA) inhibition in the LPS–antihomologous polyvalent rabbit O-antiserum test system by the LPS isolated from heterologous cultures to elucidate the correlation between LPS immunochemical activity and the set and structure of repeating units comprising the corresponding OPS.

MATERIALS AND METHODS

The subjects of the study were *P. syringae* strains pvs: *aptata* IMV 185; *atrofaciens* IMV 4394 = ATCC 4394^T, 2399, K1025, 2846, 948, 8281; *coronafaciens* IMV 9030; *glycinea* IMV L-25; *lachrymans* IMV 7591, 7595; *lupini* IMV 1234; *maculicola* IMV 381; *morsprunorum* IMV CF-4; *phaseolicola* IMV 120a, GSPB 1449; *pisi* IMV 7175; *ribicola* NCPPB 1010; *syringae* IMV 281 = NCPPB 281^T, 218, P-55; *syringae* (*cerasi*) IMV 467b, 435; *syringae* (*holci*) IMV 1055ab, 8299, 8300, 90a; *syringae* (*populi*) IMV 460; *tabaci* IMV 223, P-28, 225; *tomato* IMV 140R; *vignae* IMV 7241; and *wieringae* IMV 7923, obtained from the bacteria collection of Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine (Kyiv).

Bacteria were grown on potato agar for 20–22 h at 26–28°C. LPS were extracted from wet microbial mass with 0.85% NaCl water solution and purified with repeated ultracentrifugation (3 × 4 h, 105000 *g*) [9]. OPS fractions were separated as described elsewhere [9]. Partial acid hydrolysis of OPS to cleave selectively the terminal α-D-Fuc*f* (structures **22** and **28** in Table 1) was performed in trifluoroacetic acid (0.5% OPS solution in 1% CF_3COOH, 15–30 min, 100°C). Hydrolysis products were separated on a TSK-40 column (65 × 1.6 cm) using phenol and sulfuric acid reaction to control the separation. The high-molecular fraction was lyophilized. To selectively cleave the terminal residues of β-D-GlcNAc (structure **32,** Table 1) *N*-deacetylation–deamination was performed as described in [9]. OPS (35 mg) was dried under vacuum in the presence of P_2O_5 and dissolved in anhydrous hydrazine (1 ml) containing hydrazine sulfate (50 mg). The reaction mixture was incubated in a sealed tube for 10–20 h at 105°C and then concentrated. *N*-deacetylated OPS was isolated by gel chromatography on a TSK-40 column. The degree of α-D-Fuc*f* and β-D-GlcNAc cleavage was monitored by ^1H and ^{13}C NMR spectroscopy of the high-molecular weight fraction.

Enzyme immunoassay (EIA) reaction was performed as was described in [9]. Polyvalent immune sera were obtained against heat-killed (2.5 h, 100°C) bacterial cells.

RESULTS AND DISCUSSION

The enumerated *P. syringae* strains represent various serological groups of the classification scheme [3] which is based on the results of direct and cross reactions of agar-precipitation of the thermostable antigen obtained by the Grasset technique with whole and cross-sorbed sera against live and heated bacterial cells. The authors of [3] arranged over 300 strains into nine groups. Unpurified antigens obtained by the Grasset technique, according to our data (data not shown), are characterized by a wider spectrum of antigen determinants (including probably some of noncarbohydrate nature) than the LPSs of the corresponding strain. Moreover, the authors did not perform serotyping of the strains within serogroups. Therefore, in order to improve and clarify the classification scheme [3] we have studied serological relations between *P. syringae* strains using structurally characterized LPS preparations as antigens. Immunochemical properties were determined by comparative inhibition of EIA reaction in the test system "LPS–anti-O-serum against heat-killed (2.5 h, 120°C) bacterial cells" with homologous LPS and LPS of strains with identical or similar OPS structure. Typical representatives of each of the nine serological groups of the classification scheme [3] were used as test strains.

As follows from the data obtained (Tables 2 and 3), in all "LPS–anti-O-serum" systems native LPS preparations efficiently inhibited homologous EIA reaction (80–100%). High serological affinity (52–100%) was revealed also upon inhibition of the test system by

Table 1. Structures of repeating units identified in the OPS of *P. syringae* LPS and schematic presentation thereof

Structures of repeating units and their numbering		Chemo-type**
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(3←1)-α-D-Fuc3NAc	1*	3*L*a^{1,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(3←1)-α-D-Fuc3NAc	2*	4*L*a^{1,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ α-*D*-Fuc3NAc(1←3)⌋	3*	4'*L*a^{4,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(3←1)-α-D-Fuc3NAc	4*	4'*L*a^{1,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(3←1)-αD-Fuc3NAc ⌊(3←1)-α-D-Fuc3Nac	5*	4'*L*a^{1,3} a^{4,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→	6*	3*L*
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→	7*	4'*L*
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→	8	4*L*
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(2←1)-β-D-GlcNAc	9*	4'*L*b^{2,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(2←1)-β-D-GlcNAc	10	4'*L*b^{3,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(2←1)-β-D-GlcNAc	11	4*L*b^{2,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(3←1)-β-D-GlcNAc	12	4'*L*b^{1,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(3←1)-β-D-GlcNAc	13	4*L*b^{1,3}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(2←1)-α-D-Fuc3NAc	14*	3*L*a^{2,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(2←1)-α-D-Fuc3NAc	15*	4'*L*a^{2,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(2←1)-α-D-Fuc3NAc	16*	4*L*a^{2,2}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(4←1)-α-D-Fuc3NAc	17	4'*L*a^{2,4}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(4←1)-α-D-(1→2)-α-Fuc3NAc	18	4*L*a^{2,4}
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→ ⌊(4←1)-α-D-Fuc3NAc-(1→2)-α-D-Fuc3NAc- (1→2)-α-D-Fuc3NAc	19	4'*L*a$^{2,4+}$
→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→ ⌊(4←1)-α-D-Fuc3NAc-(1→2)-α-D-Fuc3NAc- (1→2)-α-D-Fuc3NAc	20	4*L*a$^{2,4+}$
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→	21*	3*D*
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→ α-D-Fuc*f*-(1→4)⌋	22*	3*D*c^{3,4}
→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→ ⌊(3←1)-α-D-Rha	23*	3*D*d^{3,3}
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→ α-D-Rha-(1→3)⌋	24*	4*D*d^{4,3}
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→	25	4*D*

Table 1. (Contd.)

Structures of repeating units and their numbering		Chemotype**
α–2-O-Me-D-Rha-(1→2 или 3)-α-D-Rha-(1→3)-α-D-Rha-(1→2 or 3)-α-D-Rha-(1→	26	4*D*e[1,2]
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→ 10–100%⌊(4←1)-β-D-GlcNAc	27*	4*D*b[2,4]
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→ α-D-Fuc*f*-(1→4)⌋	28*	4*D*c[4,4]
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→ ~30%⌊3-OMe ⌊(4←1)-α-D-Fuc*f*	29	4*D*c[4,4] e[2,3]
→2)-α-D-Rha-(1→3)-α-D-Rha-(1→2)-α-D-Rha-(1→3)-α-D-Rha-(1→ α-D-Fuc*f*-(1→4)⌋	30	4*D*'c[4,4]
→3)-α-L-Rha-(1→4)-β-L-Rha-(1→3)-α-D-Rha-(1→	31*	3*LD*
→3)-α-L-Rha-(1→4)-β-L-Rha-(1→3)-α-D-Rha-(1→ β-D-GlcNAc-(1→4)⌋	32*	3*LD*b[3,4]

Notes: Notes: *, Structures described with participation of the authors; **, chemotype is a schematic representation of a repeating unit structure where "3" and "4" stand for tri- and tetrasaccharide O-units of the linear rhamnan skeleton of the O-chain; L and D are the absolute configuration of rhamnose residues in the rhamnan skeleton of the OPS chain; a, b, c, d, and e represent lateral substituents in the rhamnan skeleton of the O-chain, D-Fuc3NAc, D-GlcNAc, D-Fuc*f*, D-Rha, and O-Me, respectively; [2,2, 1,2], etc.: the first numeral designates the number of the rhamnose residue at a branching site counting from the nonreducing end of the OPS chain, and the second numeral indicates the substituent localization in a rhamnose residue; [+], lateral residue is a trisaccharide of D-Fuc3NAc residues; 4*L* and 4'*L* are repeating units of L-rhamnose tetrasaccharide differing by the localization of the substituent group in position [3] or [2] of the 4th rhamnose residue, respectively; 4*D* and 4'*D* are repeating units of D-rhamnose tetrasaccharide differing in the localization of the substituent group in position [3] or [2] of the 4th rhamnose residue, respectively.

the LPS of heterologous strains belonging to the same serogroup as the strain used to prepare the test system. Meanwhile, strains within one serogroup varied in terms of their affinity to the test system strain (except for serogroup VI), which probably indicates the presence of different serotypes within a single serogroup. According to immunochemical properties of the LPS, strains of serogroups II and VII were the most heterogeneous (20–100%). Weak interrelations (0–40%) were observed upon the EIA inhibition by LPS of the strains belonging to serogroups heterologous to the test system strain.

In order to find out which structural parts of a complex LPS macromolecule induce antibody formation, a number of strains were analyzed for comparative inhibition of homologous and heterologous EIA reactions not only with native LPS, but also with the fractions of OPS, core oligosaccharide, and lipid A, obtained upon LPS hydrolysis, and with the LPS modified by selective cleavage of α-D-Fuc*f* residue in structures 22 and 28 and β-D-GlcNAc residue in structure 32 (Tables 1 and 3).

OPS fractions obtained by LPS hydrolysis in 1% acetic acid followed by gel chromatography of the carbohydrate part on a Sephadex column efficiently (62–100%) inhibited the EIA reaction in a homologous "LPS–anti-O-serum" system (Table 3). Fractions of the core oligosaccharide and lipid A of most of the strains were also serologically active. Therefore, antigen determinants located in these parts of the macromolecule may also contribute to the development of immune response toward the LPS molecule in the host organism and, along with the OPS, determine the serological relations between strains.

Comparative EIA inhibition in test systems of strains IMV 218, 8299, and 225 with native and modified homologous LPS demonstrated significant decrease or complete loss of serological activity upon selective elimination of terminal α-D-Fuc*f* residues and of the β-D-GlcNAc residue (see Tables 1 and 3). The data obtained are evidence of the exceptional importance of side substituents in the OPS rhamnan skeleton for the immunological activity of LPS macromolecules. The data also indicate that O-sera against strains IMV 8299 and IMV 225 contain no antibodies against the rhamnan chain, that is, the rhamnan skeleton is immunologically inert in the presence of lateral substituent groups. The phenomenon is the most pronounced in the case of LPS of strains IMV 225 and IMV 7591 (Table 3). While the central linear rhamnan chain of the OPS is identical in both strains (Table 1, structures **31** and **32,** respectively), strains of different pathovars are not serologically related and thus belong to different serogroups (VIII and IX) according to the scheme [3]. However, affinity between the strains was revealed upon eliminating of side residues (β-D-GlcNAc) in the OPS of stain IMV 225. The modified linear rhamnan obtained from strain IMV 225 exhibited high immunological activity (EIA inhibition by 63%) in the heterologous system of LPS–strain IMV 7591 O-serum and very lightly (19%) inhibited the homologous LPS–strain IMV 225 O-serum system. Weak reactivity is probably exerted by the antigen determinants located in the OPS chain core part.

Table 2. Serological interrelation between bacterial strains of *P. syringae* group

System of LPS and O-serum of the strain	Serogroup [3]	Strain of the LPS-inhibitor	Inhibition**, %
P. syringae pv. *syringae* IMV 281	I	281	100
		8281	100
		460	100
		381	56
		8300	52
		9030	58
	VII	140R	38
		223	13
P. syringae pv. *atrofaciens* IMV K1025	II	K1025	100
		90a	87
		467	100
		435	72
		218	54
		CF-4	42
P. syringae pv. *syringae* (*cerasi*) IMV 467	II	467	100
		90a	100
		K1025	71
		218	39
		CF-4	20
		435	82
P. syringae pv. *syringae* IMV 218	II	218	100
	III	P-55	74
	VI	120a	40
		2399	24
		8299	24
P. syringae pv. *syringae* IMV P-55	III	P-55	100
	II	218	18
	VI	120a	20
		2399	22
		8299	24
P. syringae pv. *atrofaciens* IMV 4394	IV	4394	100
		1055a	95
		2846	56
		185	79
		7923	81
		7157	94
		L-25	65
		7841	58
	V	948	5
	VIII	225	13
P. syringae pv. *atrofaciens* IMV 948	V	948	100
	IV	4394	5
		L-25	20
	VIII	225	28
P. syringae pv. *syringae* (*holci*) IMV 8299	VI	8299	100
		120a	100
		2399	100
	III	P-55	18
	II	218	24
P. syringae pv. *tabaci* IMV 223	VII	223	100
		140R	23

Table 2. (Contd.)

System of LPS and O-serum of the strain	Serogroup [3]	Strain of the LPS-inhibitor	Inhibition**, %
	I	281	31
		460	30
		381	27
		8300	23
P. syringae pv. *tabaci* IMV 225	VIII	225	100
	V	948	28
	IV	7923	0
		4394	13
		1234	15
		7157	13
		2846	13
		L-25	0
		1055a	0
		185	13
		7841	15
P. syringae pv. *lachrymans* IMV 7591	IX	7591	80
	VIII	225	0
	II	K1025	0
		467	0
		CF-4	0

Notes: * On the basis of interaction of O-sera against heat-killed bacteria with LPS preparations isolated from homologous or heterologous cultures in the IEA reaction.

** Maximum inhibition of the IAE reaction in various systems at the LPS-inhibitor concentration of 1.56–200 μg/ml. The data are averages of three or four experiments.

How do the established immunochemical characteristics correlate with the structural specificity of OPS in *P. syringae* LPS?

Analysis of the structural data [5–8] demonstrates that a number of *P. syringae* strains contain not one, but two or three, structural types of repeating units in varying proportions. The set of the repeating units identified for a particular strain represents the chemotype of its OPS. The chemotype of each strain's OPS according to the key (Table 1) is presented (Table 4). For the reader's convenience, we also provide the formula of the chemotypes where the structures of repeating OPS units are labeled with figures according to the numeration (Table 1). Among the 25 pathovars of 55 *P. syringae* strains, 25 OPS chemotypes were identified. Most of the chemotypes (16 out of 25) contain more than one repeating unit and differ in the composition of the repeating units' set.

Data analysis suggests that *P. syringae* strains may be grouped according to the characteristics of their LPS structure. No matter which of the pathovars a strain belongs to, many of them possess identical OPS structures. According to this characteristics, 42 of 55 strains fall into 15 groups each containing two to nine strains (Table 4).

Some of the repeating units' structures are found in several OPS. The identified 25 OPS chemotypes are grouped into 15 chemogroups depending on the presence of one or two common repeating units structures (see Table 4). A number of strains contain only group-specific repeating unit(s); therefore, 6 out of 15 chemogroups are isolated. Some of the strains lack repeating units that would be specific only for its corresponding chemotype, for example as in the OPS of IMV 281^T (4'*L*a^{4,3}, 4*L*a^{1,3}, 3*L*a^{1,3} chemotype; structures **1, 2,** and **3**; Tables 1 and 4), which is in agreement with its status as a reference strain.

Members of the proposed chemogroup I (Table 4) share a repeating unit (structure **1,** Table 1). Four out of six chemotypes of the group also contain a common repeating unit structure **2** (Table 1). As the OPS chemotype 4'*L*a^{1,3}a^{4,3}, 4*L*a^{1,3} (structures **2** and **5**, respectively, Tables 1 and 4) does not contain the repeating structure **1**, it was referred to chemogroup I as chemotype $I'_{2,5}$. Besides the strain-specific unit 3*L* (structure **6**), the OPS of the strain IMV 9030 possesses a structure **3** repeating unit characteristic of the $I_{1,2,3}$ chemotype (Table 4) and a 4'*L* unit (structure **7**) characteristic of chemogroup II. Thus, we refer it to chemogroups I and II as chemotypes $I''_{3,6,7}$ and $II_{3,6,7}$, respectively. Therefore, chemogroup I includes strains of different OPS chemotypes.

In strain IMV 281, an EIA inhibition test system (Table 2) by the LPS of strains with identical OPS (IMV 8281 and 460) or an OPS similar at the level of common repeating unit(s) (IMV 140R, 8300, 381, and 9030), only those strains possessing LPS identical to

Table 3. Comparative serological activity* of various structural parts of the LPS macromolecule

System of LPS and O-serum of the strain	Serogroup [3]	Inhibitor preparation isolated from the strain					
		Strain	LS	OS	Rhamnan	Core	Lipid A
P. syringae pv. *syringae* IMV 281	I	281	100	98		79	100
P. syringae pv. *atrofaciens* IMV 8281		8281	100	100		55	96
P. syringae pv. *maculicola* IMV 381		381	100	100		+	+
P. syringae pv. *syringae* (*populi*) IMV 460		460	100	100		+	+
P. syringae pv. *syringae* (*holci*) IMV 8300		8300	100	100		+	+
P. syringae pv. *syringae* (*cerasi*) IMV 467	II	467	100	100		15	35
P. syringae pv. *morsprunorum* IMV CF-4		CF-4	100	100		+	+
P. syringae pv. *syringae* (*cerasi*) IMV 435		435	100	100		+	+
P. syringae pv. *syringae* IMV 218		218	100	100	23	0	20
P. syringae pv. *atrofaciens* IMV K1025		K1025	100	62		0	57
P. syringae pv. *syringae* IMV P-55	III	P-55	100	100		4	4
P. syringae pv. *atrofaciens* IMV 4394	IV	4394	100	66		13	54
P. syringae pv. *atrofaciens* IMV 948	V	948	100	100		32	93
P. syringae pv. *syringae* (*holci*) IMV 8299	VI	8299	100	90	0	+	+
P. syringae pv. *phaseolicola* IMV 120a		120a	100	85		+	+
P. syringae pv. *tabaci* IMV 223	VII	223	100	92		58	55
P. syringae pv. *tomato* IMV 140R		140R	100	95		+	+
P. syringae pv. *tabaci* IMV 225	VIII	225	100	78	19	53	70
	IX	7591	0	0			
P. syringae pv. *lachrymans* IMV 7591	IX	7591	80	65	63	4	37
	VIII	225	0	0			

Notes: * IEA inhibition analysis data in O-sera against heat-killed bacterial cells–anti-LPS of a homologous strain.

** Maximum inhibition (%) of the reaction at the inhibitor preparation concentration of 1.56–200 μg/ml; (+), positive reaction. Averages of three or four experiments.

that of the strain IMV 281 LPS exhibited serological affinity (EIA inhibition by 100% as in the case of homologous LPS). LPS with similar OPS structures (one or two common repeating units), within the same concentration range (1.56–200 mg/ml), inhibited the reaction by 38–58% at most. Therefore, strains IMV 281, 8281, 460, 8300, 381, 9030, and 140R grouped into chemogroup I according to their OPS structure are also affined by immunochemical characteristics; based on this data, we group them into serogroup I as serotypes I_1, $I_{1,2}$, $I_{1,2,3}$, and $I_{1,2,4}$, which corresponds to their chemotypes (Table 4). Strains IMV 381 and 9030, serologically related to strain IMV 281, are referred to serogroup I as serotypes $I'_{2,5}$ and $I''_{3,6,7}$, respectively.

Weak interrelations (5–31% EIA inhibition) were observed between strains sharing only some structural fragments of the OPS chain. The rhamnan with the same side residues in the OPS chains of strains IMV 281 and 223 or the 4'*L* rhamnan in the OPS chains of strains IMV 281 and 948 LPS (see Tables 1, 2, and 4) are examples. The phenomenon suggests that the rhamnan chain is immunologically inert when shielded by side residues. Apparently, it is not the epitopes located on the OPS chain that are responsible for the weak relation between IMV 281 strain and strains IMV 223 and 948; this suggestion is supported by the activity of the core and lipid A fractions (see Table 3). Strains of serogroup I (Table 4) match their classification in the scheme [3]. The only exception is *P. syringae* pv. *tomato* IMV 140R. We may also assume that strains *P. syringae* pv. *savastanoi* OTM 519, ITM 317, and PVF 5, which were not studied in the work in terms of immunochemical characteristics of their LPS also belong to serogroup I as serotype I^1.

We referred the serologically related (EIA reaction inhibition by 56–95%, Table 2) strains of chemogroup $XI_{25,27}$ to serogroup IV under serotype $IV_{25,27}$ as did the authors of [3] (Table 4). Strains of chemogroup $XI_{25,27}$ were also referred to the serogroup under serotype $IV_{25,26}$, since their OPS share the common chain unit structure **25** (Table 1). No relation was revealed with strains IMV 948, P-28, and 225 (Table 2), which share a D-GlcNAc fragment as a side residue in the OPS with the chemogroup representatives.

Strain IMV 948 (Tables 2 and 4), a representative of the chemogroup sharing a common repeating unit

Table 4. Chemotypes of *P. syringae* LPS OPS and chemo- and serogrouping schemes on the basis of LPS structure and immunochemical properties

Pathovar and strain	OPS chemotype**	Formula***	Chemogroup and chemotype	Serogroup and serotype	Serogroup [3]
savastanoi OTM 519, ITM 317, PVF 5*	$3La^{1,3}$	1	I_1	I_1(?)	–
tomato IMV140R	$\underline{3La^{1,3}}$, $4La^{1,3}$	1, 2	$I_{1,2}$	$I_{1,2}$	VII
syringae IMV 281^T, *atrofaciens* IMV 8281, *syringae* (*populi*) IMV 460, *garcae* NCPPB 588*	$\underline{4'La^{4,3}}$, $4La^{1,3}$, $3La^{1,3}$	1, 2, 3	$I_{1,2,3}$	$I_{1,2,3}$	I
syringae (*holci*) IMV 8300	$\underline{4'La^{1,3}}$, $4La^{1,3}$, $3La^{1,3}$	1, 2, 4	$I_{1,2,4}$	$I_{1,2,4}$	
tomato GSPB 483, *maculicola* IMV381*	$\underline{4'La^{1,3}a^{4,3}}$, $4La^{1,3}$	2, 5	$I'_{2,5}$	$I'_{2,5}$	
coronafaciens IMV 9030	$\underline{3L}$, $4'L$, $4'La^{4,3}$	3, 6, 7	$I''_{3,6,7}$ $II_{3,6,7}$	$I''_{3,6,7}$ $V_{3,6,7}$	
garcae NCPPB 2708	$\underline{4L}$, $4'L$	7, 8	$II_{7,8}$	$V_{7,8}$(?)	–
atrofaciens IMV948	$4'L$, $\underline{4'Lb^{2,2}}$	7, 9	$II_{7,9}$	$V_{7,9}$	V
porri NCPPB 3545, 3365*	$\underline{4'Lb^{3,2}}$, $4Lb^{2,2}$, $4'L$	7, 10, 11	$II_{7,10,11}$	$V_{7,10,11}$(?)	–
ribicola NCPPB 1010	$\underline{4'Lb^{1,3}}$, $4Lb^{1,3}$	12, 13	$III_{12,13}$	$III_{12,13}$	–
tabaci IMV 223	$3La^{2,2}$	14	IV_{14}	VII_{14}	VII
coriandricola GSPB 2028 (W-43)	$4La^{2,2}$, $\underline{4'La^{2,4}}$	15, 16	$V_{15,16}$	$X_{15,16}$	–
garcae NCPPB 1399	$\underline{4'La^{2,4}}$, $4La^{2,4}$	17, 18	$VI_{17,18}$	$XI_{17,18}$	–
delphinii NCPPB 1879	$\underline{4'La^{2,4+}}$, $4La^{2,4+}$	19, 20	$VII_{19,20}$	$XII_{19,20}$	–
morsprunorum C28, IMV CF-4, pv. *syringae* (*cerasi*) IMV 467*	$3D$	21	$VIII_{21}$	II_{21}	II
syringae IMV 218, IMV P-55, *morsprunorum* CFBP 1650*	$3D$, $\underline{3Dc^{3,4}}$	21, 22	$VIII_{21,22}$	$II_{21,22}$	II + III
syringae (*cerasi*) IMV 435	$3Dd^{3,3}$	23	IX_{23}	$II_{21,23}$	II
atrofaciens IMV K-1025, *syringae* (*holci*) IMV 90a*	$4Dd^{4,3}$	24	X_{24}	$II_{21,24}$	II
atrofaciens IMV 7836, *glycinea* GSPB 1991, *helianthi* CFBP 2149, 1732*	$4D$, $4De^{1,2}$	25, 26	$XI_{25,26}$	$IV_{25,26}$(?)	–
aptata IMV 185, *atrofaciens* IMV 4394^T, 2846, *glycinea* IMV L-25, *lupini* IMV 1234, *pisi* IMV 7157, *syringae* (*holci*) IMV 1055a, *vignae* IMV 7241, *wieringae* IMV 7923*	$\underline{4Db^{2,4}}$, $4D$	25, 27	$XI_{25.27}$	$IV_{25,27}$	IV
atrofaciens IMV 2399, *syringae* (*holci*) IMV 8299, *phaseolicola* IMV 120a, GSPB 1489, *tagetis* ICMP 6370*	$4Dc^{4,4}$	28	XII_{28}	VI_{28}	VI
phaseolicola GSPB 1552	$4Dc^{4,4}e^{2,3}$, $\underline{4Dc^{4,4}}$	28, 29	$XII_{28,29}$	$VI_{28,29}$(?)	–
phaseolicola NPS 3121	$4'Dc^{4,4}$	30	$XIII_{30}$	$XIII_{30}$	–
lachrymans IMV 7591, NCPPB 1096*	$3LD$	31	XIV_{31}	IX_{31}	IX
tabaci IMV P-28, 225, NCPPB 79*	$3LDb^{3,4}$	32	XV_{32}	$VIII_{32}$	VIII

Notes: * Groups of strains with identical OPS structures.

** Set of repeating units of the strain(s) OPS. See Table 1 for the key to schematic description of the structures. The structure of the repeating unit dominating in the strain OPS is underlined.

*** Set of repeating unit structures in the OPS chain of the strain(s) with respect to their numbering (see Table 1); (–), not studied; (?), serological characteristics not studied.

structure **7** (Table 1), is serologically isolated. Its LPS did not inhibit the EIA reaction of strain IMV 281, although they both contain 4'*L* rhamnan in the OPS skeleton. The strain IMV 948 test system was slightly inhibited by LPS with the rhamnan chain substituted with D-GlcNAc similarly to its own chain (Tables 1, 2, and 4). Apparently, the weak relation is provided by the core oligosaccharide as a structurally similar OPS of *P. syringae* pv. *ribicola* NCPPB 1010 (see Table 4) did not exhibit affinity toward IMV 948 in immunoprecipitation reaction (data not shown) and upon interaction with MAbs [10]. The data indicate that the linear rhamnan chain is immunologically inert when shaded by side residues. We referred strain IMV 948 to serogroup V as a $V_{7,9}$ serotype (Table 4), similar to the authors of [3]. Strain IMV 9030 was also assigned to the same group as a $V_{3,6,7}$ serotype according to the presence of the structure **7** repeating unit in the OPS chain (Table 1) and based on the interrelation between the strains through their LPS in precipitation and agar-precipitation cross-reactions (data not shown). However, as strain IMV 9030 also contains structural units **3** and **6** in its OPS chain, it also belongs to serogroup I as serotype $I''_{3,6,7}$. Strain pathovars *garcae* NCPPB 2708 and *porri* NCPPB 3545 and 3365 were included in serogroup V as serotypes $V_{7,8}$ and $V_{7,10,11}$, as their OPS share a common unit (structure **7**).

Inhibition of the test systems of strains IMV 8299, 223, 225, and 7591 also supported the correlation between the data of structural and immunochemical analysis (Tables 1, 2, and 4). LPS with the OPS chemotype identical to the one of the test system strain efficiently (by 80–100%) inhibited the EIA reaction. The revealed weak interrelations (0–30%) apparently are provided by the core oligosaccharide of the LPS, which is confirmed by the absence of any relations between strain IMV 7591, with the core different from that of any other strain according to the data on interaction with MAbs [10] and structurally similar LPSs.

Strains with OPS chain chemotypes IV_{14} (IMV 223), XII_{28} (IMV 2399, 120a, and 8299) XIV_{31} (IMV 7591), and XV_{32} (IMV P-28 and 225) were assigned to serogroups VII, VI, IX, and VIII as serotypes VII_{14}, VI_{28}, IX_{31}, and $VIII_{32}$, respectively (Table 4). Strain *P. syringae* pv. *phaseolicola* GSPB 1552 was assigned to serogroup VI as serotype $VI_{28,29}$ basing on the presence of structure **28** unit in the OPS chain (Table 1).

Identical structure or presence of common repeating units in the OPS chain of strains within a chemogroup correlates with serological identity or close serological affinity in cross-reactions (see Tables 1, 2, and 4). However, in a number of reactions of EIA inhibition by identical or structurally similar LPS in the test systems of strains IMV P-55, 218, K1025, and 467, no correlation was observed between the structure and immunochemical properties of the OPS. For example, the test system of strain IMV 467 was weakly (19.8%) inhibited by strain IMV CF-4 with an identical OPS chain chemotype. Similar behavior was observed upon IMV P-55 test system inhibition by the LPS of strain IMV 218. High affinity was revealed between some strains sharing no common units in their OPS chain, e.g., in the case of inhibition of the IMV K1025 test system by the LPS of strains IMV 467, CF-4, 218, and 435 or inhibition of the IMV 467 test system by LPS of IMV 435 (see Tables 1, 2, and 4). The enumerated strains belong to serogroup II, except for IMV P-55, which belongs to serogroup III according to the scheme [3]. The specific characteristics of their LPS may be explained in different ways.

Strains characterized by different OPS chain chemotypes may produce a response in cross-reactions because of the presence of identical oligosaccharide units not detected by the structural analysis due to their low concentration. The possibility of the existence of such minor structures is supported by the repeated study of OPS structures with the same techniques, but using NMR spectroscopy of higher resolution (600 MHz). Along with the previously identified pentasaccharides (structures **2, 3,** and **4,** Tables 1 and 4) in the OPS structure of IMV 281 and 8300 [6], they contain a tetrasaccharide unit (structure **1**), and strain IMV 140R [6] together with structure **1** contains also another pentasaccharide unit (structure **2**) [11, 12]. We have revealed for the first time this type of microheterogenity in the OPS chain of the LPS of gram-negative bacteria.

Keeping this in mind, we grouped the strains IMV 218, 467, 435, CF-4, K1025, and 90a with OPS chain chemotypes $VIII_{21}$, $VIII_{21,22}$, IX_{23}, and X_{24} which belong to serogroups II and III according to the scheme [3], into serogroup II as serotypes II_{21}, $II_{21,22}$, $II_{21,23}$, and $II_{21,24}$. We suppose that repeating structure **21** is the common unit for all the serotypes of the group (see Tables 1 and 4). The absence of this structure in strains IMV K1025 and 435 according to LPS analysis may be ascribed to its low content in the OPS chains. The idea is supported by analysis of the antigen schemes of strains IMV K1025 and 90a by O-antisera crossdepletion by bacterial cells, suggesting that LPS of strain K1025 should contain an O-antigen factor absent in strain 90a (data not shown). The data also correlate with the inability of strain IMV 90a to inhibit completely the IMV K1025 test system (Table 2).

It is well-known [15] that immune response to the LPS molecule is largely determined by the structure of the terminal repeating unit located at the nonreducing terminus of the O-chain. It has been established for a number of *P. syringae* strains [11] that, in an OPS chain containing up to three chemotypes of repeating units, the latter are arranged in blocks. LPS molecules of a single strain are always heterogeneous in terms of OPS chain length. Therefore, the LPS pool of a specific strain may contain OPS chains differing by the chemotype of their repeating units, which comprise a block at the terminal nonreducing end of the OPS chain and thus may differ serologically.

Based on analysis with MAbs, the authors of [14] assigned strains IMV P-55 and 218 to an infrequent *P. syringae* serogoup O:3 cross-reacting only with the strain pv. *morsprunorum* C28 (structure **21,** Table 1). Strains IMV K2105, 90a, and 435 exerting a high affinity level to these strains were assigned to a different serogroup.

Repeating units of *P. syringae* pv. *delphinii* NCPPB 1879 OPS chain (structures **19** and **20,** Table 1) with the D-Fuc3NAc side residue differ from the others. We assigned the strain to chemogroup $VII_{19,20}$ and serogroup XII under the $XII_{19,20}$ serotype (Table 4).

The named peculiarities in OPS structure may be used to determine the specific taxonomic status of corresponding strains and pathovars.

Strains *P. syringae* pvs. *ribicola* NCPPB 1010, *coriandricola* GSPB 2028 (W-48), *garcae* NCPPB 1399, *phaseolicola* NPS 3121 of chemotypes $III_{12,13}$, $V_{15,16}$, $VI_{17,18}$, and $XIII_{30}$ were assigned to serogroups III, X, XI, and XIII as serotypes $III_{12,13}$, $X_{15,16}$, $XI_{17,18}$, and $XIII_{30}$, respectively, according to the OPS chain structure (Table 4). Each of the strains so far is the single representative of its serogroup.

Thus, comparative analysis of the immunochemical characteristics and LPS structure data revealed that serological heterogeneity of *P. syringae* strains correlates with OPS chain microheterogeneity in the bacterial LPS.

Studies of agar precipitation, hemagglutination and its inhibition, immunoelectrophoresis (data not shown), and EIA and its inhibition demonstrated that O-specific serological interrelations between strains of different serotypes exist only when their OPS chains share common repeating units rather than structural fragments, as was considered previously. In the presence of side residues, the linear OPS skeleton stays serologically inert. An approach considering identical, and not just similar, structures of repeating units was used for serogrouping of the strains for the first time.

P. syringae classification data based on the OPS structure and EIA reaction inhibition correlate with the classification scheme [3] (see Table 4). Only strains *P. syringae* pv. *tomato* IMV 140R and pv. *syringae* IMV P-55 were reclassified. Strain serotyping was performed.

There is a definite correlation between the classification scheme designed on the basis of OPS structure and the MAb reactivity [10, 14]. As in the scheme based on the MAbs reactivity, in scheme [3] and our scheme, the strains with OPS central chain containing rhamnose units of different absolute configurations are assigned to different serogroups (Table 4). Serogroups V, VII, VIII, and IX coincide completely across the three schemes.

Classification scheme [4] developed using polyclonal sera in agar precipitation reactions groups 51 *P. syringae* strains into 23 serogroups. The scheme does not divide serogroups into serotypes. The use of strains with OPS of unknown structures hampers the correlation with other schemes. However, in the case of strains described in [3] and in the present work, there is a definite correlation.

Besides *P. syringae* classification schemes based on their immunospecificity, there is strain division into genomotypes according to the data of DNA–DNA hybridization and ribotyping [2] and into pathovars according to host-plant specificity [1]. It should be mentioned that genomotyping was performed, generally, only for the reference strains of each pathovar, while OPS of the strain within the same pathovar may be of different structures. For example, *P. syringae* pv. *atrofaciens* strains are distributed among five chemogroups (Table 4).

In the case of *P. syringae* spp. pathovars *savastinoi*, *porri, phaseolicola, lachrymans*, and *morsprunorum*, a correlation was revealed between the OPS structure and the host-plant specificity. However, no correlation was found for most of the strains. For example, strains of *P. syringae* pv. *atrofaciens* may differ in OPS chain composition, as well as in rhamnose absolute configuration in the major chain. On the other hand, strains with identical OPS structure may be referred to different pathovars as, for example, are the representatives of serogroups I, IV, and V (Table 4).

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