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EXPERIMENTAL ARTICLES

Some Characteristics of *Pseudomonas syringae* pv. *maculicola* Dissociants

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Abstract—*Pseudomonas syringae* pv. *maculicola* dissociants producing colonies of different morphotypes were found to possess similar biochemical and serological properties but different virulence to the host plant. The heterogeneous extracellular and intracellular lipopolysaccharide–protein complexes of the dissociants differed in their chemical composition and biological activity towards test plants.

Key words: *Pseudomonas syringae* pv. *maculicola*, dissociation, virulence, lipopolysaccharide, lipopolysaccharide–protein complex.

The bacterium *Pseudomonas syringae* (van Hall 1902) has been comprehensively studied by our group for a long period of time with respect to its chemical composition, structure, taxonomy, serology, the biological activity and function of lipopolysaccharides (LPSs), and the mechanisms of interaction in the host plant–bacterial parasite system. Bacteria used in such investigations should possess stable species characteristics. Preliminary studies showed, however, that the collection culture *P. syringae* pv. *maculicola* (McCulloch 1911) Young *et al.* 1978 is heterogeneous with respect to the morphology of the colonies produced. This prompted us to comparatively study the characteristics of the cell-surface constituents (lipopolysaccharides, proteins, and their complexes), as well as some physiological, biochemical, and virulent properties, of different colonial morphotypes of *P. syringae* pv. *maculicola.*

In the present paper, we report the results of these studies.

MATERIALS AND METHODS

The strain *Pseudomonas syringae* pv. *maculicola* 381 used in this work was obtained from the collection of living cultures at the Department of Phytopathogenic Bacteria, Zabolotnyi Institute of Microbiology and Virology. This strain was isolated from cauliflower in 1957 and maintained at room temperature on potato agar (PA) by regular subculturing.

To test strain homogeneity, it was plated onto PA, incubated at 27°C for 5 days, and examined for the morphology of the grown colonies. The relevant physiological, biochemical, and serological properties of the selected variants of strain 381 were studied by conventional methods [1]. Antisera were produced by injecting rabbits five times with increasing concentrations of live cells at 5-day intervals (OH antiserum) or dead cells at 2- to 3-day intervals (O antiserum). The blood was collected on the 8th to 13th day after the last antigen injection. Live cells (OH antigens) were obtained from a 24-h-old *P. syringae* pv. *maculicola* culture. Dead cells (O antigens) represented the *P. syringae* pv. *maculicola* culture that was killed by heating at 100°C for 2.5 h.

Cell virulence was estimated under field conditions by placing a drop of a bacterial suspension containing 10^9 cells per milliliter sterile tap water onto a cauliflower leaf and then damaging the leaf surface under the drop with a trident needle. The results of this test, which was conducted in 5–7 replicates, were recorded after 5 to 7 days on a five-point scale.

The allergenicity of the selected bacterial variants was tested by the aseptic subepidermal injection of a suspension containing 10^7 cells per milliliter sterile tap water into a tobacco leaf. The results were recorded after 1 to 3 days of incubation.

To obtain bacterial biomass, *P. syringae* pv. *maculicola* cells were grown on PA at 27°C for 24 h. Cells were extracted with 0.85% NaCl and precipitated by centrifugation at 5000 *g* for 30–40 min at 4°C. Intracellular lipopolysaccharide–protein (ILPS-P) complex was obtained by the second extraction of precipitated cells with 0.85% NaCl [2]. Extracellular lipopolysaccharide–protein (ELPS-P) complex was obtained from the first NaCl extract as described earlier [3]. The extract was brought to 50% saturation by adding powdered $(NH_4)_2SO_4$ and kept in a refrigerator for about 20 h. The precipitate was collected by centrifugation at 5000 *g* for 30 min. ILPS-P and ELPS-P preparations were dissolved in a minimal volume of water, dialyzed against distilled water, and lyophilized. The prepara-

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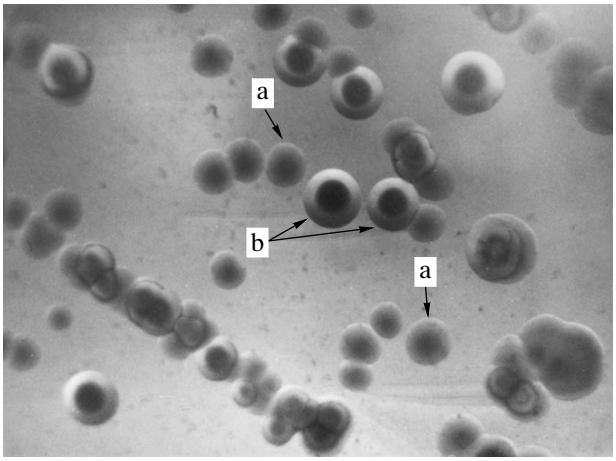

The dissociation of *P. syringae* pv. *maculicola* strain 381 into variants (a) 381-vir and (b) 381-avir.

tions were studied without purifying them additionally from protein components.

The monosugar composition of the preparations was determined as described elsewhere [2, 3]. Amino compounds were detected using an AAA-339 amino acid analyzer [4].

The biological activity (phytopathogenicity and allergenicity) of the ILPS-P and ELPS-P complexes was assayed by injecting their solutions (1 mg/ml) with a sterile syringe into the fruits of growing tomato plants or the subepidermis of white cabbage and tobacco leaves. The control plants were administered with sterile water instead of lipopolysaccharide solutions. The biological activity was detected by the appearance of watery spots on tomato fruits, cabbage leaf tuberosity, and tobacco chlorosis.

RESULTS AND DISCUSSION

The scheduled subculturing of *P. syringae* pv. *maculicola* 381 in 1998 on PA showed that this strain underwent dissociation, which manifested itself in the formation of differing sectors and segments on the growing colonies. The multiple subculturing of strain 381 allowed us to obtain two dissociants (or variants), which were stable for 6–7 months.

Table 1. The agglutination cross-reaction titers of antisera to the O and OH antigens of *P. syringae* pv. *maculicola*

Antiserum	Antigen	Dissociant	
		381-avir	381-vir
OH (381)	OH	12800	12800
	O	12800	12800
O (381)	OH	25600	25600
	O	6400	12800
O (381-avir)	OH	25600	25600
	O	25600	25600
O (381-vir)	OH	25600	25600
	O	12800	12800

One bacterial variant, designated later as 381-vir, produced colonies typical of the original strain 381, namely, shiny, circular (4–5 mm in diameter), translucent, slightly crenate colonies with dense cone-shaped centers (see figure).

The other variant, designated later as 381-avir, produced colonies atypical of the original strain 381, namely, circular (3–5 mm in diameter) colonies with dense dome-shaped centers clearly visible in transmitted light. The colony margins were wide, thin, even, and transparent. These colonies resemble fried eggs when observed in transmitted light and could hardly be detached from the agar surface with a loop.

Both variants (variant 381-avir to a greater degree) were also susceptible to dissociation. When subcultured, variant 381-avir either reverted to the original phenotype or dissociated into several unstable variants. Variant 381-avir dissociated, both in vitro and in vivo, throughout autumn and the first half of winter more severely than over a period from the second half of the winter to the end of summer.

Both variants were gram-negative aerobes producing weak turbidity and yellowish to greenish fluorescence when grown in nutrient broth. The variants fermented glucose, sucrose, and (slowly) mannitol, sorbitol, and glycerol. Maltose, lactose, dulcitol, and salicin were not utilized. Litmus serum was alkalified. Boiled milk was coagulated. Reactions for the formation of indole and hydrogen sulfide, reduction of nitrates, and the liquefaction of gelatin were negative. Biochemically, variant 381-avir slightly differed from variant 381-vir in that it fermented glucose and sucrose more slowly, produced a smaller amount of fluorescent pigment, and alkalified litmus serum more intensively. Cells of both variants were arranged singly, in pairs, or in chains, whose length was greater in variant 381-avir.

Virulence studies showed that, like the original strain 381, variant 391-vir is allergic to tobacco plants and highly virulent to cauliflower plants occurring at different developmental stages, from plants with 3–4 mature leaves to plants with curd ovary. The infection of plants with variant 391-vir under field conditions led to the appearance of butyrous necroses, which then developed into dry brownish tinted necroses with dark margins. The leaf tissue around the necroses soon turned yellow. In the course of time, the affected leaf became entirely yellow and dry. On the other hand, the allergenicity of variant 381-avir to tobacco plants was low, and this variant was not virulent to the host plant.

According to data available in the literature, the dissociation of phytopathogenic pseudomonads usually gives rise to rough (R-type) colonies. However, Konovalova described as many as six different colonial mor-

Table 2. Relative content of various LPS complexes in cells of the *P. syringae* pv. *maculicola* 381 dissociants

Dissociant	Biomass, g dry cells	ILPS-P, %	ILPS-P*, %	ELPS-P, %	ELPS-P*, %
381-vir	8.1	16.1	2.5	0.17	1.6
381-avir	4.9	7.5	1.6	1.4	0.5

Table 3. The agglutination cross-reaction titers of antisera to various lipopolysaccharide complexes of the original and dissociated *P. syringae* pv. *maculicola* strain 381

Complex	Agglutination titer							
	OH antiserum (381) +		O antiserum (381) +		O antiserum (381-vir) +		O antiserum (381-avir) +	
	Dissociant							
	381-vir	381-avir	381-vir	381-avir	381-vir	381-avir	381-vir	381-avir
ELPS-P	10000	10000	1000	1000	10000	10000	10000	10000
ELPS-P*	1000	1000	1000	1000	1000	1000	10000	10000
ILPS-P	10000	10000	1000	1000	10000	10000	10000	10000
ILPS-P*	10000	–	1000	–	1000	–	1000	–

Note: The symbol "–" indicates that titer was not determined because of the turbidity of the reaction medium.

photypes of the bacterium *Pseudomonas holci* Kendrick (*P. syringae* Van Hall) [5]. Generally, bacteria of the genus *Pseudomonas* are fairly variable, even with respect to taxonomically important traits, such as the shape of cells, the presence of flagella, and cell motility [6–9]. It should be noted that, during the subculturing of some collection strains of *P. syringae* pv. *atrofaciens* and during the microbiological analysis of affected wood tissues, we observed colonies resembling those of variant 381-avir but disregarded them. The possibility cannot be excluded that other researchers also observed such avirulent bacterial forms but disregarded them as well. The transition of bacteria to the R form was reported to either increase [6], or not to influence [8, 10, 11], or to decrease (sometimes to zero) [12, 13] bacterial virulence. The inconsistency of these data can be accounted for by the heterogeneity of natural bacterial populations with respect to virulence, as was shown for the bacterium *P. syringae* pv. *coronofaciens* [14].

In spite of minor differences in the properties of the dissociants observed in agglutination reactions with OH and O antigens (live cells and cells killed by heating at 100°C for 2.5 h), the dissociants were found to be serologically identical. Both dissociants showed the same or very close titers in agglutination reactions with the whole-cell antisera to homologous and heterologous cells and with the OH and O antisera against the original (undissociated) *P. syringae* pv. *maculicola* strain 381 (Table 1).

Such a high level of serological relatedness of the variants suggests that their O antigens (lipopolysaccharides) must be similar. The yields of the ILPS-P and ELPS-P complexes of the dissociants differed consider-

Table 4. Hyperplasia on cabbage leaves induced by the subepidermal injection of various lipopolysaccharide complexes of the *P. syringae* pv. *maculicola* dissociants

Dissociant	Complex	Degree of hyperplasia	
		early-maturing Dymerskaya cabbage	late-maturing Amager cabbage
381-vir	ELPS-P	±	++
381-vir	ILPS-P*	+	–
381-vir	ILPS-P	–	+++
381-avir	ELPS-P	+	+
381-avir	ILPS-P*	+–	+
381-avir	ILPS-P	+	–

Note: The symbol "–" indicates the absence of hyperplasia. The symbols "+-," "+," "++," and "+++" indicate the presence of hyperplasia with increasing intensities.

Table 5. The monosugar composition of various lipopolysaccharide complexes of the *P. syringae* pv. *maculicola* dissociants

Monosugar	Monosugar content, % of the total sugars							
	381-vir				381-avir			
	ILPS-P	ILPS-P*	ELPS-P	ELPS-P*	ILPS-P	ILPS-P*	ELPS-P	ELPS-P*
Rhamnose	27.5	42.5	11.2	12.0	41.1	12.3	18.4	9.2
Ribose	10.6	9.9	0.3	2.5	–	17.0	1.1	1.6
3-Acetamido-3-deoxyfucose	20.6	2.2	2.1	Traces	14.0	10.4	2.1	4.4
Glucosamine	1.9	5.1	3.2	1.7	9.2	0.7	18.2	7.6
Glucose	3.1	17.2	7.8	70.5	13.3	23.6	5.1	63.9
Mannose	–	0.7	–	–	–	6.4	–	–
Galactose	–	0.4	59.4	7.9	1.1	1.9	29.0	7.2
Heptose	5.0	9.5	–	0.8	2.2	–	–	–
X	29.9	11.0	9.7	4.6	17.3	3.3	12.2	9.2
X_1	–	–	–	–	–	24.5	–	–
X_2	–	–	2.94	–	–	–	–	–

Note: *X*, X_1, and X_2 denote unidentified sugars.

ably (Table 2): variant 381-vir yielded considerably more ILPS-P and less ELPS-P than variant 381-avir.

As opposed to our earlier experiments, in the present experiments, the dialysis of the ILPS-P and ELPS-P preparations against distilled water resulted in the precipitation of considerable fractions of these preparations (the precipitates were designated as ILPS-P* and ELPS-P*, respectively) (Table 2). Analysis showed that all four preparations (ILPS-P, ELPS-P, ILPS-P*, and ELPS-P*) were serologically active in agglutination reactions with the OH and O antisera against the original culture and with the O antisera against variants 381-vir and 381-avir (Table 3). These data indicate that all the preparations represent O antigens and possess common antigenic determinants. The low serological activity of the preparations allows the suggestion to be made that their lipopolysaccharides have short *O*-specific chains or represent R-type lipopolysaccharides.

The preparations caused the formation of watery spots on infected tomato fruits and induced hyperplasia (leaf tuberosity) on infected cabbage plants. The biological activity of particular preparation depended on the cabbage variety (Table 4). Tobacco chlorosis was induced only by the lipopolysaccharides of variant 381-vir. The higher biological activity of lipopolysaccharide preparations extracted from the variant 381-vir cells indicates that bacterial lipopolysaccharides play an important part in the process of plant infection.

The ILPS-P complexes of both bacterial variants were dominated by rhamnose, which is the typical monosaccharide of the LPSs of phytopathogenic pseudomonads. These complexes also contained glucose, glucosamine, and an unidentified sugar (Table 5). The content of rhamnose and glucose in the ILPS-P complex of variant 381-avir was considerably greater than in the ILPS-P complex of variant 381-vir. The monosugar compositions of the precipitated ILPS-P* and native ILPS-P complexes differed both qualitatively and quantitatively.

The monosaccharides of the native ELPS-P complexes were dominated by rhamnose and galactose. Instead of galactose, the precipitated ELPS-P* complexes were dominated by glucose. The ELPS-P complex of variant 381-vir differed from that of variant 381-avir in that the former contained a twofold greater amount of galactose and a smaller amount of glucosamine. The contents of minor sugars were also different.

Among the amino compounds (Table 6) of the LPSs of both bacterial variants, we detected alanine (the typical amino acid of LPSs in pseudomonads), asparagine, threonine, serine, glycine, valine, leucine, histidine, ornithine, ethanolamine, some minor amines, and the amino sugars glucosamine and galactosamine. The ILPS-P and ELPS-P complexes of both bacterial variants differed from each other not only in monosugar composition but also in amino acid composition. Therefore, in spite of their identical serological activity towards O antisera, the ELPS-P and ILPS-P complexes of either bacterial variant are not identical. The precipitation of ELPS-P* and ILPS-P* complexes during the dialysis of native ELPS-P and ILPS-P complexes indicates that the native complexes are heterogeneous. This is in agreement with the data of Varbanets, who reported on the heterogeneity of the cell-wall lipopolysaccharides of *P. syringae* as long ago as 1980 [15]. The structural heterogeneity of O antigens was shown for the *P. syringae* strains of serogroup I [16, 17] and the bacterium *Burkholderia* (*Pseudomonas*) *solanacearum* [17, 18].

Table 6. Amino compounds (amines) and their content in various lipopolysaccharide complexes of the *P. syringae* pv. *maculicola* dissociants

Amine	Amine content, % of the total amines							
	381-vir				381-avir			
	ILPS-P	ILPS-P*	ELPS-P	ELPS-P*	ILPS-P	ILPS-P*	ELPS-P	ELPS-P*
Phosphoethanolamine	0.6	0.4	–	0.7	4.0	–	1.6	–
Asparagine	8.2	9.0	2.2	7.8	5.7	8.7	6.9	7.9
Threonine	3.1	3.4	1.2	3.1	2.1	3.5	2.6	3.2
Serine	4.5	4.7	3.5	4.6	3.6	4.5	4.4	5.6
Glutamic acid	7.5	8.4	2.8	7.6	4.7	7.6	5.7	7.6
Proline	0.3	0.3	–	0.2	0.2	0.2	0.2	0.3
Glycine	7.0	7.6	3.6	7.4	5.8	7.6	6.4	8.4
Alanine	7.9	8.7	3.1	7.4	8.5	9.3	6.5	6.9
Valine	3.0	3.0	0.8	2.5	2.3	3.1	1.9	2.3
Methionine	0.3	0.1	–	–	0.4	0.5	–	–
Diaminopimelic acid	0.3	0.3	–	–	0.6	1.0	–	–
L-Leucine	2.1	1.9	0.4	0.6	1.6	2.0	1.3	1.6
Leucine	6.4	6.5	1.3	5.4	4.5	5.8	4.5	5.4
Tyrosine	2.0	1.8	–	1.6	1.6	2.1	1.3	1.6
Phenylalanine	3.0	2.6	–	2.0	2.0	2.8	1.7	2.1
Glucosamine	1.9	1.9	1.2	1.7	9.2	3.0	3.3	1.4
Galactosamine	0.7	0.4	–	0.3	3.6	0.7	0.9	–
Histidine	6.9	6.0	5.4	6.1	7.1	4.3	9.8	5.1
Ornithine	0.2	0.1	0.8	0.2	0.4	0.2	0.3	0.5
Lysine	5.1	5.1	1.4	4.4	2.7	4.3	3.4	4.2
Ethanolamine	0.9	1.0	1.9	1.3	4.9	1.5	1.6	0.8
Arginine	4.0	4.1	1.2	3.5	2.5	3.2	2.2	2.6
X_1	–	0.3	–	0.6	0.5	–	0.5	–
X_2	0.1	0.2	–	–	–	0.3	0.4	–
X_3	0.3	0.2	–	–	0.6	0.4	–	–
X_4	0.3	0.2	–	–	–	0.3	–	–
X_5	–	0.1	–	–	–	–	–	–
X_6	2.0	1.8	–	1.7	1.4	2.2	1.7	1.7
X_7	0.7	0.2	–	0.5	–	–	–	0.6
X_8	–	0.1	–	–	–	–	–	–
X_9	–	–	–	–	–	0.1	–	–
X_{10}	–	–	–	–	0.1	–	–	–

Note: X_1 through X_{10} denote unidentified amines.

To conclude, the ELPS-P and ILPS-P complexes of *P. syringae* pv. *maculicola* strain 381 are variable and heterogeneous. The dissociants 381-vir and 381-avir of this strain are similar in their biochemical and serological properties but differ in the morphology of their colonies, virulence, and the characteristics of their ELPS-P and ILPS-P complexes. The structural characteristics of the *O*-specific polysaccharides of these dissociants are the subject of our next report.

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