Separation of Strains of *Pseudomonas syringae* pv. *tomato* into Serovars by Three Serological Methods

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ABSTRACT

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Serological variation within *Pseudomonas syringae* pv. *tomato* was observed when 26 strains from widely separated areas of the United States and Canada were tested by Ouchterlony double diffusion (ODD), microagglutination (MA), and indirect immunofluorescence (IIF) methods. A strong correlation existed between two of the tests for placing the strains into two serovars. One serovar (designated serovar I) consisted of 20 strains, and serovar II contained five strains. One strain did not fit into either serovar. When the MA and IIF techniques were used, separation into serovars was only possible using cross absorbed antisera with other strains of *P. syringae* pv. *tomato*. ODD was not a definitive test for separation of the strains into two serovars. ODD was useful for identifying those strains in serovar I; however, inconclusive results were obtained with strains we classified as serovar II. The existence of serovars should be considered in attempts to develop serological methods for rapid detection and identification of *P. syringae* pv. *tomato*.

Additional key words: *Lycopersicon esculentum*, transplant diseases.

In recent years, bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young et al (2) (hereafter referred to as *P. tomato*) has become a serious problem both in tomato (*Lycopersicon esculentum* Mill.) transplant production fields in southern Georgia and in major fruit-producing areas of the northern United States where the plants are shipped (6). Since diseased transplants are a potential source of inoculum in northern areas, the use of stringent certification procedures for transplants is important. Rapid and accurate diagnosis is required before certification decisions can be made since bacterial speck can sometimes be confused with other diseases (5,16) or noninfectious disorders. Results of earlier studies (10,12,14) suggest the usefulness of serological methods for identifying members of the genus *Pseudomonas*, although the presence of common antigens and cross-reactions have caused problems (14). Despite these inherent problems, the possibility of rapidly establishing identification by using serological techniques alone or in combination with other procedures justifies continued research and refinement of the techniques. In efforts to develop a serological method for detecting and identifying *P. tomato*, we found evidence through indirect immunofluorescence staining that there was considerable serological variation among strains. Lucas and Grogan (10) emphasized the need to determine the extent of serological variability for a nomenspecies before serological methods can be used for identification.

The purpose of this work was to determine whether serovars of *P. tomato* exist and to evaluate three types of serological tests for separating strains into serovars.

MATERIALS AND METHODS

Bacterial strains. Twenty-six strains of *P. tomato* from various habitats and locations in the United States were used in the study (Table 1). All strains were tested in the greenhouse and were found to be pathogenic on Chico III tomato plants. The strains were maintained between studies on slants of nutrient-yeast-dextrose agar (23 g nutrient agar, 5 g yeast extract, 10 g glucose, and 1 L water, pH 6.8) at 6 C.

Antisera production. Four strains (PT2, PT4, PT10, and PT15) were used to immunize rabbits to produce antisera. The strains were grown in petri plates on medium B of King et al (7) for 24 hr at 25 C. Bacterial cells were suspended in saline (40 ml of 0.85% NaCl per plate) and the resulting suspension was autoclaved for 30 min at 121 C (J. D. Taylor, *personal communication*). The autoclaved suspension was centrifuged for 10 min at 12,000 *g* at 4 C, and the pellet was washed twice in saline. The cells were resuspended in saline and adjusted to 10^{10} cells per milliliter. Frozen nonautoclaved whole cells (10^{10} cells per milliliter) were also used as immunizing antigens for one isolate (PT2). One milliliter of each antigen preparation was mixed 1:1 with Freund's incomplete adjuvant and injected intramuscularly into each of the two New Zealand white rabbits at weekly intervals for 3 wk. One week after the final injection, the rabbits were bled at the marginal ear vein. Bleeding was continued at weekly intervals for 4–6 wk. The blood was refrigerated overnight and fractionated by centrifugation at 1,800 *g* for 15 min. The antisera were collected, recentrifuged for 5 min to eliminate red blood cells, and stored at −20 C until used.

Each of the three antisera (PT2, PT4, and PT15) was cross absorbed with the immunizing antigens of each of the three strains in all possible combinations. Cultures of the bacteria were grown in nutrient broth on a rotary shaker (200 rpm) for 24 hr at 25 C. The cells were harvested by centrifuging for 10 min at 12,000 *g*. The resulting pellet was suspended in 10 ml of antiserum diluted 1:10 with saline, the suspension was incubated 24 hr at 25 C, and centrifuged 15 min at 12,000 *g*. The supernatant was recovered and the procedure was repeated until no observable microagglutination of the absorbed antiserum and homologous antigen occurred, which indicated that complete absorption had been accomplished.

Serological testing. Microagglutination (MA), indirect immunofluorescence (IIF), and Ouchterlony double diffusion (ODD) methods were compared. MA tests were conducted by mixing equal amounts (one drop to one drop) of six serial dilutions (ranging from 1:4 to 1:4,096) of each antiserum and a bacterial suspension (10^9 cells per milliliter) in a sterile plastic petri plate. Prepared plates were incubated for 20–30 min at 25 C, and the drops were observed with a Cycloptic stereoscope (AO Instrument Co., Buffalo, NY 14215) for an agglutination reaction. The greatest

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dilution at which agglutination occurred was considered to be the titer of the antiserum for the strain involved.

The IIF staining procedure described by Schaad (17) was used. Slides were prepared from cell suspensions containing 10^7 cells per milliliter. Fourfold dilutions (ranging from 1:4 to 1:4,096) of antisera with saline were made to determine the titer at which cells stained most clearly. Prepared slides were examined at ×250 with a Wild Dual-Illumination Microscope (Wild Heerburgg Instrument, Inc., Heerburgg, Switzerland) equipped with an HBO-200 mercury vapor lamp (burner) and an oil-immersion condenser. The BG12 filter and a barrier filter in the OG1 position were used to provide a suitable wavelength (transmission range above 530 nm).

ODD tests were conducted as described by Lucas and Grogan (11), except that Difco purified agar replaced Ionagar No. 2. Plates were incubated for 2 wk at 4 C to accentuate band development. In most studies, suspensions of living cells (10^{10}/ml) were used to react with the antiserum. In one study, sonicated bacterial suspensions were used and in others a lipopolysaccharide (LPS) component was used. The sonicated suspension was prepared by treating a suspension of living cells (10^{10}/ml) with a Biosonik IV sonicator (Ultrasonic Power Corp., Trenton, NJ 08628) at maximum intensity at 4 C until the suspension became opalescent. LPS was prepared from cells grown at 25 C in nutrient broth for 24 hr on a rotary shaker at 200 rpm. Cells were harvested by centrifuging the suspension at 4 C for 10 min at 12,000 *g* and crude LPS was obtained by the phenol extraction method (19). The extracted LPS was suspended in water (5 mg/ml) for placing in the antigen wells. Heated fractions were obtained by autoclaving at 121 C for 30 min.

RESULTS

All three serological techniques demonstrated antigenic variation among the 26 strains of *P. tomato* tested (Tables 2–6). By using absorbed antisera with MA and IIF, strains were separated into two serovars. The largest group (serovar I) consisted of 20 strains. Five strains were placed in serovar II and one strain could not be placed in either serovar. There was good agreement between the two serological methods, MA and IIF, for placing the strains into the two serovars.

Serological variation was evident in the IIF studies since titer differences occurred when the various strains were tested with a particular antiserum preparation (Table 2). Most of the strains had higher titers when tested with PT4 and PT15 antisera than with PT2 and PT10 antisera. However, six strains (PT1, PT2, PT6, PT10, PT15, and PT20) had either equal or higher titers when tested with PT2 and PT10 antisera as when tested with PT4 and PT15 antisera. Considerable titer variation also occurred among strains tested by the MA technique (Table 3). With most strains, titers were higher when tested with PT4 and PT15 antisera than with PT2 and PT10 antisera. An exception occurred when the PT2 and PT10 strains were tested with PT4 and PT15 antisera.

The use of cross-absorbed antisera with both the IIF (Table 4) and MA techniques (Table 5) made it possible to separate the strains into distinct groups. In the case of IIF, the occurrence of slight variability in reaction patterns with absorbed antisera made grouping more difficult than with MA. Cross-absorption of PT2 antisera and PT2 cells after four absorptions was not completely successful in eliminating antibodies specific for PT2. One group of 12 strains (PT3, PT5, PT7, PT11, PT12, PT13, PT14, PT15, PT16,

TABLE 1. Origin of strains of *Pseudomonas tomato* used in tests to determine serological variation

Strain number	Original strain designation	Source	
		Worker[a]	Location
PT1[b]	Field 1	S. M. McCarter	Georgia
PT2	Field 3	S. M. McCarter	Georgia
PT3	Field 4	S. M. McCarter	Georgia
PT4	Field 6	S. M. McCarter	Georgia
PT5	Field 7	S. M. McCarter	Georgia
PT6	Field 8	S. M. McCarter	Georgia
PT7	PT80-11	R. D. Gitaitis	Georgia
PT8	PT80-16	R. D. Gitaitis	Georgia
PT9	179	S. M. McCarter	Georgia
PT10	375	S. M. McCarter	Georgia
PT11	289	S. M. McCarter	Georgia
PT12	85	S. M. McCarter	Georgia
PT13	232	S. M. McCarter	Georgia
PT14	Cotyledon 1	S. M. McCarter	Georgia
PT15	BL13	D. L. Coplin	Ohio
PT16	BL36	D. L. Coplin	California
PT17	BL51	D. L. Coplin	New Jersey
PT18	BL62	D. L. Coplin	California
PT19	BL72	D. L. Coplin	California
PT20	78-2	R. E. Stall	Florida
PT21	44(997)	M. Sasser	Delaware
PT22	*P. tomato* #1	W. G. Bonn	Canada
PT23	P4083	W. G. Bonn	Canada
PT24	PDA32	S. H. Kim	Pennsylvania
PT25	PDA36	S. H. Kim	Pennsylvania
PT26	PDA24	S. H. Kim	Pennsylvania

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[b]All strains were isolated from tomato foliage or fruit except PT9-PT14, which were from tomato seed.

TABLE 2. Immunofluorescence titers[a] of four antisera when tested against whole cell preparations of *Pseudomonas tomato*

Strain	Antisera			
	PT2	PT4	PT10	PT15
PT1	4,096	4,096	256	256
PT2	4,096	256	256	256
PT3	64	4,096	16	1,024
PT4	16	256	4	256
PT5	16	1,024	64	256
PT6	1,024	1,024	256	256
PT7	4	1,024	4	1,024
PT8	16	256	16	256
PT10	4,096	16	256	256
PT15	4	256	256	256
PT16	256	1,024	16	1,024
PT17	16	256	64	256
PT18	64	4,096	64	256
PT19	64	256	256	256
PT20	1,024	256	256	256
PT21	16	4,096	256	256
PT22	16	ND[b]	16	256
PT23	16	ND	64	256

[a]Titer of last observable fluorescent staining of bacterial cells.
[b]Not determined.

TABLE 3. Microagglutination titers[a] of rabbit antisera prepared against autoclaved whole cells of *Pseudomonas tomato*

Strain	Antisera			
	PT2	PT4	PT10	PT15
PT1	256	256	64	1,024
PT2	256	4	256	16
PT3	4	256	16	1,024
PT4	4	1,024	16	1,024
PT10	256	4	256	4
PT15	16	1,024	16	1,024
PT16	16	1,024	16	1,024
PT18	4	256	16	1,024
PT19	16	1,024	ND[b]	1,024
PT20	256	1,024	256	1,024
PT21	16	1,024	4	1,024

[a]Titer of last observable agglutination of bacterial cells.
[b]Not determined.

TABLE 4. Immunofluorescence reactions with antisera (AS) prepared against autoclaved whole cells of *Pseudomonas tomato* and absorbed by whole cells (WC) of *Pseudomonas tomato* strains

Strain	Serovar designation	PT2 AS absorbed by WC of strains			PT4 AS absorbed by WC of strains			PT15 AS absorbed by WC of strains		
		PT2	PT15	PT4	PT2	PT15	PT4	PT2	PT15	PT4
PT3, PT5, PT7, PT11, PT12, PT13, PT14, PT15, PT16	I	–[a]	–	–	+	–	–	+	–	–
PT1, PT24	I	+	–	–	+	–	–	+	–	–
PT4, PT17, PT21	I	–	+	–	+	–	–	+	–	–
PT8, PT18	I	–	–	–	+	+	–	+	–	–
PT6	?[b]	+	–	–	+	–	–	+	–	–
PT19	?[c]	+	–	+	+	+	–	+	–	–
PT2, PT9, PT10	II	+	+	+	–	–	–	–	–	–
PT20, PT26	II	+	+	+	+	–	–	–	–	–

[a] + = fluorescent staining of bacterial cells; – = no fluorescent staining of bacterial cells.
[b] With this pattern, PT6 would be placed in serovar I for this test. However, using ODD data, PT6 in actuality is not in serovar I.
[c] With this pattern, PT19 could not be placed in either serovar.

TABLE 5. Microagglutination reactions with antisera (AS) prepared against autoclaved whole cells of *Pseudomonas tomato* and absorbed by whole cells (WC) of *P. tomato* strains

Strain	Serovar designation	PT2 AS absorbed by WC of strains			PT4 AS absorbed by WC of strains			PT15 AS absorbed by WC of strains		
		PT2	PT15	PT4	PT2	PT15	PT4	PT2	PT15	PT4
PT1, PT3, PT4, PT5, PT7, PT8, PT11, PT12, PT13, PT14, PT15, PT16, PT18, PT19, PT21, PT22, PT23, PT24, PT25	I[a]	–	–	–	+	–	–	+	–	–
PT17	I	–	–	–	+	–	–	+	–	+
PT6	?[b]	–	–	–	+	–	–	+	–	–
PT2, PT9, PT10, PT26	II	–	+	+	–	–	–	–	–	–
PT20	II	–	+	+	+	–	–	–	–	–

[a] – = no precipitation band close to antigen well; + = precipitation band close to antigen well.
[b] With this pattern, PT6 would be placed in serovar I. However, using ODD data, PT6 in actuality is not in serovar I.

PT22, PT23, and PT25), considered to be serovar I, had identical reaction patterns. Eight other strains (PT1, PT4, PT6, PT8, PT17, PT18, PT21, and PT24) gave reaction patterns very similar to the 12 mentioned above. These eight strains reacted with PT4 and PT15 that was cross absorbed with cells of PT2 so that they were placed in serovar I. Five strains (PT2, PT9, PT10, PT20, and PT26) were placed in serovar II because they had closely related reaction patterns that differed from the other groups when absorbed antisera were used. They reacted with PT2 antisera when absorbed with the three cell preparations. Strain PT19 gave a different reaction and could not be reasonably placed in either serovar based on the IIF tests with absorbed antisera.

In the MA tests with absorbed antisera, 21 strains (serovar I and PT6) gave identical or similar reaction patterns (Table 5). The five strains of serovar II (PT2, PT9, PT10, PT20, and PT26) had reaction patterns in common, but differed markedly from the other 21 strains. Strain PT20 had a slightly different reaction pattern than the other five, but was placed in serovar II. Generally, reaction patterns with absorbed antisera were less variable in the MA than in the IIF tests.

Data from the ODD tests were useful for detecting serological diversity and distinguishing one serovar (Table 6). PT4 and PT15 antisera reacted with most of the strains, forming a precipitin band close to the antigen well. In no instance was spur formation observed. Precipitin bands were not formed with strains PT2, PT6, PT9, PT10, PT20, and PT26 when tested with PT4 and PT15 antisera. Since PT4 and PT15 antisera did not react with PT6 in ODD tests, PT6 was not placed in serovar I even though, in MA and IIF tests with cross-absorbed antisera, its reaction pattern was characteristic of a serovar I strain. PT2 and PT10 antisera did not react in ODD with any of the strains tested and hence the test was of no value for determining the serovars of the remaining strains in serovar II. PT2 antiserum prepared to nonautoclaved cells formed identical bands with serotype I and serotype II antigens. With LPS preparations, serovar I antiserum reacted with LPS prepared from serovar I (strains PT15 and PT21), but not from serovar II (strain PT2). Serovar II antiserum did not react with LPS prepared from

TABLE 6. Ouchterlony double diffusion reactions of whole cells of strains tested against antisera to autoclaved cells of *Pseudomonas tomato*

Strain	Serovar designation	Antisera			
		PT2	PT4	PT10	PT15
PT1, PT3, PT4, PT5, PT7, PT8, PT11, PT12, PT13, PT14, PT15, PT16, PT17, PT18, PT19, PT21, PT22, PT23, PT24, PT25	I	–[a]	+	–	+
PT6	ND[b]	–	–	–	–
PT2, PT9, PT10, PT20, PT26	ND	–	–	–	–

[a] – = no precipitation band close to antigen well; + = precipitation band close to antigen well.
[b] Based on these results, these strains could not be placed in a serovar classification.

serovar I (strains PT15 and PT21), but formed a weak reaction with LPS from serovar II (strain PT2).

Based on the three serological tests, PT6 was the only strain not placed in a serovar, five strains (PT2, PT9, PT10, PT20, and PT26) were placed in serovar II, and the remaining 20 strains were placed in serovar I.

DISCUSSION

Our results provide further evidence that the phytopathogenic pseudomonads are a serologically diverse group (3,8,10,11,14,15). With agglutination tests Perlasca (15) observed considerable heterogeneity among strains of *P. syringae* from stone fruits. Lovrekovich et al (8), using the gel diffusion method and heat stable antigens, classified 14 strains of *P. syringae* (including *P. morsprunorum*) into eight serovars. In a similar study, Otta and English (14) established 10 serovars among 450 strains of *P. syringae* from 30 host plants, but could not correlate place of origin with a specific serovar. Efforts to establish distinct serovars within pathovars of *P. syringae* have produced mixed results. Guthrie (4) could not establish serovars within *P. syringae* pv. *phaseolicola* due to a high level of homogeneity among strains. However, Lucas and Grogan (10,11), using gel diffusion tests, identified three distinct serovars within *P. syringae* pv. *lachrymans*. By using IIF and MA, we were able to establish two rather distinct serovars within 26 strains of *P. tomato* from various locations in the United States and Canada. Our serovar grouping of the strains is presented in Table 5. Difficulty in fitting one of our strains, such as PT6, into the two serovars is a clear indication of the existence of other serovars. However, considering the diverse sources of our strains, we feel that most strains of *P. tomato* in the United States can be classified into our two serovars with the largest number in serovar I. The existence of serovars within *P. tomato* must be considered in efforts to develop serological methods for rapid detection and identification of *P. tomato* in tomato transplants being considered for certification.

The use of three serological methods allowed a comparison of the methods for establishing distinct serovars within *P. tomato*. Titer variation between strains as observed with MA and IIF is a good indication that serological variation exists; however, as observed by Yarkus and Schaad (20), it is not a useful means of separating strains into serovars. MA and IIF were useful for recognizing serovars when absorbed antisera were used. IIF reactions with absorbed antisera were more variable than MA tests for grouping isolates. However, the reaction patterns with absorbed antisera by both methods correlated closely.

The gel diffusion method was ineffective in detecting differences between serovar I and serovar II strains with antisera prepared to autoclaved or frozen cells of serovar II. MA, on the other hand, was effective for separating the two serovars when similarly prepared serovar II antiserum was used. Possibly the specific antigen is weakly antigenic and not detectable by gel diffusion. Gel diffusion studies indicated that the specific reaction in serovar I, which is observed close to the antigen well of serovar I strains only, is evidence for a heat-stable antigen present in crude LPS and is similar to that observed with serovars I and II of *P. syringae* pv. *lachrymans* (10,11). With serovar II, the specific reaction close to the antigen well was not observed when autoclaved cells were tested or antiserum prepared to autoclaved cells was used. This agrees with the reactions of rough-form strains of *P. syringae* pv. *lachrymans* observed by Lucas and Grogan (10). Serovar II antiserum prepared to frozen cells formed a band close to the antigen wells of serovar I and serovar II strains. Thus, serovar II antiserum was ineffective for distinguishing between serovars.

Gel diffusion methods have generally been used for serotyping phytopathogenic bacteria (1,8–14,18,20). Yarkus and Schaad (20) used cross-absorbed antisera in gel diffusion tests to separate *Erwinia chrysanthemi* into serovars. Our work suggests that MA and IIF, if used with carefully absorbed antisera, may be more sensitive than gel diffusion for serotyping. In some cases, a combination of methods may reveal associations that neither method used alone would indicate. For example, a combination of gel diffusion and MA tests showed the true position of strain PT6. Testing with MA or IIF alone placed the strain in serovar I, but the lack of a precipitin band close to the antigen well in ODD tests with serovar I antiserum was clear evidence that PT6 was not a serovar I strain. Based on MA and IIF with cross-absorbed antisera, PT6 was also not a serovar II strain. Thus, the use of MA and IIF in combination with ODD provided adequate proof that PT6 was antigenically unique. Neither test alone would have confirmed this, but together they proved to be highly effective for serotyping.

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