Serological Analysis of *Pseudomonas syringae* pv. *tomato*

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ABSTRACT

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Antibodies were developed to the somatic, flagellar, cytoplasmic, and extracellular antigens of *Pseudomonas syringae* pv. *tomato*. Precipitin tests were of little diagnostic value since antibodies to the cytoplasmic and extracellular antigens cross-reacted with antigens of other bacteria and plant extracts. Conversely, agglutination tests were stable, sensitive, and differential. Long-term immunization increased titers to the somatic and flagellar antigens to 16,000 and reduced cross-reactivity. Elimination of polysaccharides from the immunogen and from the test antigen further reduced cross-reactivity.

Additional key words: bacterial speck, immunofluorescence, polysaccharides.

Pseudomonas syringae pv. *tomato* (Okabe 1933) Young, Dye and Wilkie 1978, is the causative organism of bacterial speck of tomato (*Lycopersicon esculentum* Mill.). Bacterial speck symptoms are sometimes difficult to differentiate from those of bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge 1920) Dye 1978b (1,22). Since both of these microbes have a global distribution (11) and the incidence of bacterial speck is increasing (2,11), a quick diagnostic test is required. The biochemical and physiological tests for the identification of phytopathogens (5) remain unsatisfactory for the identification of *P. syringae* pv. *tomato*.

Serological analysis has been applied to *Xanthomonas vesicatoria* (7,16,18,24) and to *P. syringae* (19,21) in general. This report is a characterization of the antisera to the soluble and insoluble antigens of *P. syringae* pv. *tomato* in an attempt to identify those antigens and antisera most useful for the development of a serological test to help identify the bacterial pathogen. Three serological methods, double diffusion, bacterial agglutination, and immunofluorescence were used to evaluate the antisera and their specificity.

MATERIALS AND METHODS

Bacterial strains. Two strains of *P. syringae* pv. *tomato*, PT7 (ATCC 10862) and PTD, were used as the sources of antigens. Strain PTD was originally isolated from infected tomato plants in California. Fourteen strains of *P. syringae* pv. *tomato* and 62 of saprophytic pseudomonads were obtained from D. Coplin (Ohio Agricultural Research and Development Center, Wooster). Before being used in the experiments, all isolates were subcultured three times and examined for purity each time. They were all obligately aerobic, Gram-negative rods that produced a fluorescent pigment on King's medium B, were negative for cytochrome oxidase and starch hydrolysis, and utilized trehalose but not glucose. All virulent cultures induced a hypersensitive reaction in tobacco (14) and typical 1- to 3-mm-diameter punctate lesions with a chlorotic halo on tomato leaves (4,19). All cultures were preserved by lyophilization. Also, all cultures were subcultured monthly onto Lowe agar (12): proteose peptone, 3 g/L; $MgSO_4 \cdot 7H_2O$, 50 mg/L; $FeCl_3 \cdot H_2O$, 5 mg/L; and K_2HPO_4, 200 mg/L (pH 7.0). Cultures were grown on yeast extract-dextrose-calcium carbonate agar (YDC) (28) or on a yeast extract-salts medium (YSM), which contained yeast extract, 5 g/L; NaCl, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 200 mg/L; K_2HPO_4, 0.5 g/L; and $NH_4H_2PO_4$, 0.5 g/L.

Isolates of the following bacteria were obtained from the culture collection in the Biology Department, University of Windsor: *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Proteus mirabilis*, *Escherichia coli*, *Serratia marcescens*, *Enterobacter aerogenes*, *Salmonella enteriditis*, and *Acinetobacter calcoaceticus*.

Antigens and antisera. Whole-cell suspensions were obtained from inoculated broths of YSM that were incubated on a rotary shaker (100 rpm) for 48 hr at 25 C. The broths, 500 ml/1L flask, were inoculated with 10^8 cells from an actively growing culture. After incubation, the cultures were centrifuged (8,000 *g* for 15 min) and the pellets were washed three times with phosphate-buffered saline (0.01 M, pH 7.0) (PBS) before being resuspended to a final concentration of approximately 10^{11} cells per milliliter. Concentrations were determined turbidometrically at 650 nm. The washed cells were used as a source of the various antigens. To extract the O antigen, washed cells were resuspended to 10^9 cells per milliliter and heated to 100 C for 2.5 hr. After centrifugation at 8,000 *g* for 30 min, the pellet was resuspended in 95% ethanol and extracted for 4 hr at 37 C. The pellet was recovered by centrifugation and extracted twice with acetone. The acetone extract was air-dried and resuspended in PBS to the original volume (13). To preserve the flagellar or H antigen, 5 ml of formalin was added to each liter of broth culture. The formalinized cultures were incubated at 25 C overnight without agitation before being centrifuged gently (2,000 *g* for 30 min).

To obtain soluble cellular antigens, washed cells from 6 L of medium were resuspended in 10 ml of PBS, placed in a French pressure cell, precooled to 4 C, and then subjected to a pressure of 845 kg/cm^2 (12,000 lb/in.^{2}). The disrupted cells, checked microscopically for breakage, were diluted to 100 ml with PBS and centrifuged at 48,000 *g* for 1 hr. The soluble cellular antigens were decanted and frozen; the cell wall fraction was washed three times with PBS. The supernatants of spent broth cultures were used as soluble extracellular antigens and were frozen until used.

Rabbit IgG gamma chains were purified from whole serum. First

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the IgG was purified by ammonium sulfate fractionation and ion exchange chromatography (6) on diethylaminoethyl cellulose, which had a capacity of 0.94 meq/g (Sigma Chemical Co., St. Louis, MO 63178). Soluble gamma chains were obtained by dissociation in 0.15 M NaCl and 0.1 M 2-mercaptoethanol at pH 8.0. After 4 hr at room temperature, the solution was made 0.2 M in iodoacetamide and dialyzed against 0.15 M NaCl. The reduced and alkylated gamma chains were separated from the light chains by gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden) (8).

Production of antisera. Antibodies were developed to the various soluble or insoluble antigens for both PTD and PT7. Pairs of New Zealand white rabbits were injected with autoclaved cells, cell walls, somatic antigens (O antigens), flagellar antigens (H antigens), and the soluble cellular and extracellular antigens. The effects of a short-term and a long-term immunization schedule were compared. In the short-term series, animals were injected intravenously with 10^{11} cells or equivalent every 3 days for 3 wk and then exsanguinated 7 days later. In the long-term series, animals were injected intramuscularly with 10^{11} cells emulsified in Freund's complete adjuvant (Difco, Detroit, MI). The injection was repeated 4.5 mo later and the animals were bled 1.5 mo afterwards. The blood was allowed to clot; the clot was removed, and sodium azide (0.02%, w/v) was added to the serum. The serum was frozen until required.

Rats were injected intramuscularly with an emulsion of equal parts of Freund's complete adjuvant (Difco) and gamma chains (1 mg/ml) of rabbit IgG. The rats were reinjected with a similar dose after 1 mo and bled 1 mo later.

All antisera were purified to IgG by ammonium sulfate fractionation and ion exchange chromatography on diethyl amino ethyl cellulose (6).

Serological methods. Double immunodiffusion as described by Ouchterlony (6,20) was performed on microscope slides overlaid with barbital-buffered (0.01M, pH 8.2) agar. Each 25 × 75-mm slide was overlaid with 3.5 ml of molten agar, and 3-mm-diameter wells were punched at 7-mm centers in the agar after it had cooled and solidified. Slides were incubated in a humidity chamber at 24 C and observed daily for 1 mo when resolution was optimal.

Agglutinations were performed in Cooke microtiter plates (Dynatech Laboratories, Alexandria, VA 22314). A twofold dilution series of each serum (50 μl) was performed in duplicate in PBS. A suspension of washed cells (2×10^8 cells per milliliter) then was added to each well to bring the total volume to 150 μl. Plates were incubated overnight at 24 C and the titers were recorded the next day. Titers were stable for 24–72 hr. Titers less than $\log_2 5$ were considered insignificant.

Indirect immunofluorescence was performed as described earlier (27) and labeled cells were viewed by epifluorescence. Briefly, smears of *P. syringae* pv. *tomato* were fixed for 10 min with acetone, rinsed in PBS, and flooded with unlabeled antibodies. Thirty minutes later, the smears were rinsed, flooded with labeled anti-rabbit gamma chains, and incubated for an additional 30 min. The smears were washed and mounted under a coverslip with 0.5 M carbonate-buffered glycerol (pH 7.2). For each antiserum a block test was performed to establish the optimal concentrations of unlabeled and conjugated antiserum. The unlabeled antisera were effective from 1–100 μg/ml.

Originally, several commercial preparations of fluorescein isothiocyanate (FITC)-labeled anti-gamma chains were compared. Later, rat anti-gamma chain was labeled with fluorescein and purified as described by Sternberger (26). The molar ratio of fluorescein:antibody was 1.8:1 in this later preparation. This preparation gave optimal resolution at 15 μg/ml. Under these conditions nonspecific staining was minimal.

RESULTS

Soluble antigens. Immunodiffusion showed that strains PTD and PT7 were antigenically identical, since antigens from these strains only formed lines of identity whenever they were diffused against a given antiserum. Three precipitin lines were observed when the soluble cellular antigens were diffused against their respective antisera, whereas five other soluble antigens were extracellular and were detected in the supernatants of spent broth cultures. The antibodies to these soluble extracellular antigens were reactive with the soluble antigens of other bacteria. Precipitin lines of identity were observed with the supernatants of cultures of *Klebsiella*, *Salmonella*, *Escherichia*, *Enterobacter*, *Pseudomonas*, and *Xanthomonas* spp. The soluble antigens of field isolates that were avirulent in the pathogenicity test also formed several lines of identity with the soluble antigens of *P. syringae* pv. *tomato*. When cell envelopes of washed cells of avirulent strains were diffused against antisera to the extracellular antigens, two or three precipitin lines were observed, suggesting that some of the extracellular antigens associate with the cell wall.

Several immunodiffusion experiments were performed in an attempt to detect soluble antigens of *P. syringae* pv. *tomato* in infected tomato leaves. Seven different cultivars of tomato (Chico 111, Ottawa 78, H2653, H1630, H1409, H1706, and Peru) were inoculated with *P. syringae* pv. *tomato* and lesions were allowed to develop. Infected and uninfected leaves were ground separately in a blender and the water extracts that were clarified by centrifugation were used as sources of soluble antigen. The presence or absence of precipitin lines was recorded after the extracts were diffused against the various antisera. Precipitin lines were formed with the extracts of uninfected leaves of Chico 111, Ottawa 78, H1409, and H1630. The extracts of only one cultivar, H2653, formed no precipitin lines when uninfected leaves were used as the antigen, but it did form a single precipitin line when infected leaves were used. No precipitin lines were formed by extracts diffused against normal sera.

Insoluble antigens. The ability of the antisera to agglutinate strains of PTD and PT7 is illustrated in Table 1. Clearly, all of the components of both PTD and PT7 were highly immunogenic. None of the antisera discriminated between the strains, suggesting that PTD and PT7 are antigenically very similar. The average agglutination titers were fourfold higher after long-term immunization than after short-term immunization. The titer for antibodies to flagella was at least 32-fold higher than flagellar antibodies produced by a short-term immunization schedule.

Not only did the longer injection schedule increase the agglutination titers but the specificity was increased also. Each antiserum was titrated for its ability to agglutinate different Gram-negative bacteria. Table 2 demonstrates that, although the antisera all had high titers for strains PTD and PT7, no titers greater than $\log_2 5$ were recorded for any of the other bacteria. By comparison, when a similar series of titrations were performed with analogous antisera obtained after the short-term immunization schedule, 18% of the tests resulted in titers greater than $\log_2 5$ and reactions occurred with all genera tested except *K. pneumoniae* and *S. marcescens*. Approximately 8% of the agglutination titers were higher than those for PTD and PT7.

Indirect immunofluorescence confirmed the specificity of the

TABLE 1. Agglutination titers ($\log_2$) of antisera to *Pseudomonas syringae* pv. *tomato* strains PTD and PT7

Test cultures	Whole cells	Somatic antigen	Flagellar antigen	Cell wall
Short-term immunization				
Antiserum to PTD				
PTD	8,7[a]	7,8	8,9	6,8
PT7	10,7	9,8	11,10	8,9
Antiserum to PT7				
PTD	9,8	11,7	8,9	ND
PT7	9,12	9,12	11,9	ND
Long-term immunization				
Antiserum to PTD				
PTD	8,11	10,7	13,13	5
PT7	8,10	10,6	11,11	5
Antiserum to PT7				
PTD	10,12	ND	14,14	5
PT7	10,10	ND	14,14	5

[a] Average of at least two agglutination titers expressed as $\log_2$. Each value represents a separate antiserum.

rabbit antibodies to the somatic and flagellar antigens. When cells were coated first with anti-O, only the cell wall fluoresced, whereas exposure to anti-H labeled just the flagella. Commercial preparations of labeled anti-gamma chain were largely unsuccessful. The molar ratio of fluorescein to antibody was often so high that nonspecific binding predominated. Only one commercial preparation (Bionetics, Kensington, MO 20795) and the experimentally prepared antiserum had molar ratios of fluorescein:antibody such that nonspecific binding was not detectable.

A series of agglutination titers on bacteria isolated from the soil further confirmed the specificity of the agglutinating antibodies. Sixty-two isolates of avirulent pseudomonads were obtained from the soil and from tomato leaves. All of these isolates gave a titer of less than $\log_2$ 5 when tested against any of the antisera from the long-term immunization schedule. Fourteen virulent field isolates of *P. syringae* pv. *tomato* were examined with the antisera to the O and H antigens of PTD and PT7 and all of these gave high agglutination titers with these antisera (Table 3). Considerable variation in titer was observed with different antisera and between isolates.

All of the agglutination tests were performed on cultures grown on YSM, and under these conditions little or no capsular material was formed. When *P. syringae* pv. *tomato* was grown on a medium containing D-glucose, such as YDC, copious amounts of extracellular polysaccharide were formed. This capsule did not interfere with the titer of the antisera for any of the isolates of *P. syringae* pv. *tomato*, but when bacteria other than *P. syringae* pv. *tomato* (see Table 2) were grown on YDC medium, 18 of 111 tests were positive. When the same bacteria were grown on the YSM, none of the 111 tests had a significant titer. These data suggest either that the capsule of *P. syringae* pv. *tomato* is similar to that of other Gram-negative species or that some antigen that is common to the other bacteria is produced in sufficient amounts to induce agglutination only when glucose is present.

TABLE 2. Agglutination titers ($\log_2$) of antisera to *Pseudomonas syringae* pv. *tomato* strains PTD and PT7

Culture	Antiserum to			
	Whole cells	Somatic antigen	Flagellar antigen	Cell wall
P. syringae pv. *tomato* PT7	10[a]	9	12	5
P. syringae pv. *tomato* PTD	10	9	12	5
P. aeuruginosa	3	3	3	3
P. fluorescens	2	1	1	2
Enterobacter aerogenes	4	3	3	2
Acinetobacter	3	3	2	3
Serratia marscens	2	1	2	2
Klebsiella pneumoniae	1	0	1	1
Salmonella enteriditis	3	2	3	2
Proteus vulgaris	5	5	5	5
Proteus mirabilis	3	2	3	1
Escherichia coli	2	2	1	4

[a] Average agglutination titers of three to four similar antisera.

TABLE 3. Agglutination titers ($\log_2$) of field isolates of *P. syringae* pv. *tomato*

Isolate	Antiserum to	
	Somatic antigen	Flagellar antigen
PDT	10,7[a]	13,13,14
PT7	10,6	11,11,14
A1	10,6	10,10,9
A2	11,9	12,12,10
A3	10,11	9,11,9
A4	10,8	11,10,11
A5	10,7	10,10,10
A6	10,6	11,11,11
A10	9,6	12,11,12
A11	8,4	9,8,11
A12	9,6	12,12,13
A13	9,7	11,11,11
A15	10,7	12,11,13
A16	10,7	9,7,10
A17	10,8	10,10,10
A18	10,7	11,11,11

[a] Average of at least two agglutination titers expressed as $\log_2$. Each value represents a separate antiserum.

DISCUSSION

Lovrekovich and Klement (15) were the first to take advantage of immunodiffusion and demonstrate the presence of soluble antigens that were specific for a phytopathogen. Since then, many researchers have used immunodiffusion, with varying degrees of success, to identify or differentiate phytopathogens (25). The preliminary data in this report suggest that there are several soluble antigens of *P. syringae* pv. *tomato* but that they are of little value in differentiating pathogens from saprophytes. Many of these soluble antigens cross-react with those of other Gram-negative species or with the tissue extracts of various cultivars of tomato. Quantitative immunoelectrophoresis could be used to quantify the degree of cross reactivity with other bacteria and with tissue extracts to establish if there are any soluble antigens that are specific for *P. syringae* pv. *tomato*. It would then remain to be determined if the specific antigens are produced in detectable quantities during infection.

However, the cross-reactivity with other pseudomonads may be exploited to establish further the taxonomic status of *P. syringae* pv. *tomato* among the pseudomonads. Previous taxonomic classification efforts have relied upon nutritional and biochemical characteristics (17,23).

Although the indirect immunofluorescence test is extremely sensitive, the degree of nonspecific binding of many of the commercially labeled preparations negates this sensitivity in favor of the more stable agglutination test. To overcome the limitations of the indirect immunofluorescence test, either labeled preparations must be screened to identify those with little nonspecific binding or the labeled antibodies should be purified by solid-phase affinity chromatography.

These data show that antisera to the somatic and flagellar antigens of *P. syringae* pv. *tomato* can be of considerable diagnostic value if they are used cautiously. As early as 1947, Elrod and Braun (9,10) demonstrated that removal of polysaccharide decreased serological cross-reactivity of phytopathogens. In this series of experiments the effects of capsular polysaccharides were reduced in two ways. Polysaccharides were removed before antigenic stimulus by heating the cells or by extraction. Polysaccharide formation was avoided prior to agglutination by culturing the cells on a glucose-free medium. The combined use of these procedures eliminated a cross-reactivity rate of 18%.

Purified cell walls were less immunogenic than autoclaved whole cells, somatic antigens, or flagellar antigens, especially during the long-term immunization schedules (Table 1).

The short-term immunization schedule is similar to that of Bosshard-Heer and Vogelsanger (3), who produced antisera specific for *P. syringae* pv. *tomato*. Here, although the shorter schedule gave reasonable titers, the rate of cross-reactivity was unacceptably high. When the antibody response was allowed to mature, the observable cross-reactivity was reduced considerably.

The data suggest a single serogroup of *P. syringae* pv. *tomato*, but such an interpretation must be made cautiously. Antisera were developed to two strains only and were tested against isolates of limited geographic distribution. The antisera to these prototypes are being tested against isolates of *P. syringae* pv. *tomato* from around the world to discover how many serogroups actually exist.

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