Serotypes of Xanthomonas vesicatoria Unrelated to Its Pathotypes

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Florida Experiment Station Journal Series Paper No. 4622, Institute of Food and Agricultural Sciences.
Accepted for publication 3 April 1973.

ABSTRACT

Two serotypes, based on the presence or absence of certain precipitin bands in gel-diffusion plates, were designated among 72 isolates of *Xanthomonas vesicatoria*. These indicators were the one to three arclike bands formed close to antigen wells by nonheated antigens of either serotype against its homologous-type serum that were generally lacking with serum of heterologous type. Antisera of types I and II formed fast diffusible components with heated antigen preparations. With type I sera and heated type I antigens, these components formed a thick precipitin band, whereas heated type II antigens gave a weak precipitin reaction. Such distinction in the fast diffusible components could not be seen with type II serum; both types formed similar bands. However, the two types were distinguished by their specific agglutinin reactions. The fast diffusible components of types I and II were associated with soluble bacterial cell-surface compounds. There was no correlation between serology and pathology of *X. vesicatoria* isolates. The tomato and pepper isolates included both serotypes; thus, the tomato and pepper isolates could not be distinguished on the basis of serological tests. Also, the ability of isolates to hydrolyze starch, their resistance to streptomycin, or age in culture had no correlation with the serotypes.

Phytopathology 63:1260-1265

Additional key words: serological differences, host specificity, pepper, tomato.

There have been attempts to differentiate isolates of *Xanthomonas vesicatoria* (Doidge) Dows. from pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) on the basis of their host of origin, pathological and biochemical reactions, serology, and phage sensitivity. Burkholder & Li (1) found differences in physiology and pathology of pepper and tomato isolates. Pepper isolates were unable to hydrolyze starch; whereas, tomato isolates could do so. Doolittle & Crossan (6) found distinct host specificity to tomato or pepper among at least some of the tomato and pepper isolates. Using a gel-diffusion method, Lovrekovich & Klement (13) separated 11 pepper isolates from 22 tomato isolates. Furthermore, by phage sensitivity and starch hydrolysis tests, the tomato isolates were divided into two groups. Morton et al. (18) distinguished a typical tomato isolate from a typical pepper isolate by gel-diffusion tests alone. However, Gardner & Kendrick (8) could not distinguish isolates from pepper and tomato since they were pathologically identical and were found to be closely related on the bases of phage susceptibility and bacteriological determinative tests (7).

Serological tests have been useful in the study of phytopathogenic bacterial species. Notable among such studies are those of Lucas & Grogan (16) and Otta & English (19). By testing large numbers of *Pseudomonas* isolates that were representative of the species, these authors have shown antigenic heterogeneity within the species. They recognized a number of serotypes among *P. lachrymans* and *P. syringae* isolates.

This investigation was an attempt to verify earlier reports of serological differences between *X. vesicatoria* isolates from pepper and tomato (13, 18), and to correlate the three pathotypes of *X. vesicatoria* (4) with antigenic patterns. It was hoped that this study of the antigens of isolates of *X. vesicatoria* would enable us to devise specific serological tests for the pathogen. A brief account of this work has already been reported (3).

MATERIALS AND METHODS.—*Sources and maintenance of X. vesicatoria isolates.*—Of 72 isolates of *X. vesicatoria* used in this study, 45 were isolated from tomato, 23 from pepper, two from nightshade (*Solanum nigrum* L.), and one each from *Physalis angulata* L. and potato (*Solanum tuberosum* L.). Sixty-one were obtained from diseased plants in Florida during the years 1961-71; five from the Florida Department of Agriculture and Consumer Services, Gainesville; four from the International Collection of Phytopathogenic Bacteria, University of California, Davis; and one each from Guadaloupe and Hawaii (the latter collected by A. A. Cook). All cultures were maintained in a lyophilized condition. For use in this study, isolates were streaked on nutrient agar and single colonies were picked for transfers to a mineral-base broth (MBB) [$(NH_4)H_2PO_4$, 0.1%; KCl, 0.02%; $MgSO_4 \cdot 7H_2O$, 0.02%; glucose, 1.0%; and yeast extract, 0.1%]. The cultures were grown in this broth at 30 C for 36 to 48 hr for pathogenicity tests and serology.

Pathogenicity tests.—Pathogenicities of *X. vesicatoria* isolates were tested mainly on plants of one cultivar of tomato ('Floradel'); two cultivars of pepper, 'Early Calwonder' (ECW) and 23-1-7; and one cultivar of tobacco (F_2C_1). The syringe inoculation method (10) was used. The inocula for pathogenicity screening consisted of 10^8 cells/ml suspended in sterile saline. Control plants were inoculated with sterile saline without bacteria. The plants for pathogenicity tests were grown in a glasshouse at ambient temperature, 29 ± 4 C. The isolates that

caused necrosis within 24 hr were considered avirulent and those that caused necrosis after 24 hr were considered virulent. Using this method, Cook & Stall (4) demonstrated three pathotypes among virulent *X. vesicatoria* in Florida. Pathotype 1 was pathogenic to tomato only; 2 was pathogenic to tomato and ECW; and 3 to tomato, ECW, and 23-1-7.

Bacterial antigens.—Bacterial cells were centrifuged from the MBB medium at 10,000 *g* and washed twice with saline before use. The bacterial antigens for immunization of rabbits were prepared by sonicating 30 ml of approximately 10×10^{10} cells/ml in phosphate buffer at pH 7.2. Phosphate buffer consisted of 0.05 M KH_2PO_4-K_2HPO_4 to which were added 0.14 N NaCl and 0.001 M $MgSO_4 \cdot 7H_2O$ to make phosphate-buffered saline (PBS). The cells were sonicated intermittently for 4 min at 0-10 C and at the maximum intensity with a Biosonik III (Bronwill Scientific Co.) sonicator. The sonicated suspensions, without centrifugation, were used as antigens in immunization. The protein content of antigens was estimated by the Lowry test (15). Both sonicated and whole-cell antigens were compared in agar gel double-diffusion tests. The latter consisted of whole-cell suspensions of bacteria (14×10^8 cells/ml) in PBS. For heat treatment of antigens, whole cells of bacteria (in PBS at 14×10^8 cells/ml) in screw-cap tubes were immersed in a boiling water bath for 2 hr. The heat-treated antigens along with nonheated whole-cell antigens were compared in gel diffusion tests.

Antisera.—Antisera to seven isolates of *X. vesicatoria* were produced in New Zealand white rabbits 2.2-3.5 kg (5 to 8 lbs) that had not been immunized previously. Two rabbits were immunized per bacterial isolate. Normal serum was obtained from each rabbit prior to immunization. Sonicated antigens were adjusted to protein concentrations of 2 mg/ml for intramuscular injections and 500-750 μg/ml for intravenous and intraperitoneal injections. For intramuscular injections, the antigens were mixed with an equal volume of Freund's incomplete adjuvant. The following schedule of immunization was used: On the first day, an intramuscular injection was administered with 0.5 ml of antigen. From the second day to the eighth, seven intraperitoneal injections were given, starting with 0.25 ml of antigens and increasing the volume daily by 0.25 ml until a maximum of 1 ml was reached. After three days, a booster dose of antigens was given on the twelfth day; 1 ml through the intraperitoneal route and 0.5 ml each through the intravenous and intramuscular routes. Animals were bled from marginal ear veins or by cardiac puncture three days after the last injection. Serum samples were stored in small quantities and without additives at −20 C. When more serum samples were needed, the preimmunized animals were given one intravenous injection, and were bled three days later. The concentration of antigens for intravenous injections was found to be critical, since high concentration of antigens killed rabbits. Generally, 500 to 750 μg protein per ml of antigens proved safe. The seven isolates used for production of antigens were 68-1, 69-24, and 70-7 from pepper; and 69-13, 69-16, 70-8, and E3 from tomato.

Agar gel double-diffusion tests.—Double-diffusion tests were performed with 0.6% Colab No. 2 Ionagar in 0.01 M PBS at pH 7.2 and 0.04 ml of 80% phenol per 100 ml of the medium served as a preservative. Nine ml of this medium was poured into 9-cm diam sterile plastic petri plates. Well patterns, 4-mm between antiserum and antigen wells and 6 mm between antigen wells, were cut with an Auto-Gel® cutter (Grafar Corporation, Detroit).

Agglutination test.—A bacterial agglutination test was used to estimate the titers of sera and the antigenic relationship of isolates. For this test, washed, intact cells of isolates were used at a concentration of 2×10^8 cells/ml in saline. Antisera were diluted with saline to obtain a series of 1:2 to 1:16, 384 dilutions. Normal serum and saline controls were included in this series. Antigens and antisera were mixed at equal volumes in Kahn tubes and incubated at 56 C for 2 hrs. The titer of the serum was the highest dilution that gave visible agglutination. The homologous titer values of sera were determined also by using Colab latex (20). The antigens used in this method were obtained from supernatant fractions of bacterial sonicates centrifuged at 20,200 *g*. The antigens were adjusted to a protein concentration of ca. 500 μg/ml. The rest of the procedure was similar to the bacterial whole-cell agglutination. While comparing titer values of heterologous sera, the bacterial whole-cell agglutination was used.

RESULTS.—*Pathogenicity tests.*—Pathogenicity of isolates varied considerably (Table 1). All but five of the 72 isolates of *X. vesicatoria* induced a susceptible pathogenic reaction in tomato and a hypersensitive reaction in tobacco, and were considered virulent on the basis of their pathogenicity to tomato. Among the nonpathogenic isolates were three from tomato and one each from nightshade and *Physalis*. One of the nonpathogens induced no visible reaction; whereas, the other four caused weak and atypical reactions on the three hosts that were neither hypersensitive nor susceptible. Thirty-five of 67 isolates were pathogenic to the ECW cultivar of pepper. Sixteen of these were also pathogenic to the 23-1-7 cultivar. No isolate was pathogenic to 23-1-7 and nonpathogenic to ECW. Among 45 isolates originally from tomato, 11 were pathogenic to ECW, including two that were also pathogenic to 23-1-7. Of the 23 isolates from pepper, 13 were pathogenic to 23-1-7 in addition to ECW and tomato. One isolate from potato was pathogenic to both pepper cultivars and to tomato.

Resistance of isolates to streptomycin, and their ability to hydrolyze starch, were tested. Generally, there was no correlation between the host of origin of isolates and these two characteristics.

Serology.—Titers of antisera determined by whole-cell and latex agglutination methods were generally close. The homologous titers ranged from 512 to 2,048; higher titer values were obtained with

TABLE 1. Grouping of *Xanthomonas vesicatoria* isolates on the basis of host of origin, pathogenicity, pathotype, and serotype (the figures represent the number of isolates in each category)

Host of origin	No. of isolates tested	Pathogenic to			Nonpathogenic isolates	Serotype[c] under pathotype[d]							
		Tomato	ECW[a]	23-1[b]		Nonpathogenic		1		2		3	
						Serotype I	II	I	II	I	II	I	II
Tomato	45	42	11	2	3	1	1	27	4	8	1	1	1
Pepper	23	23	23	13	0	0	0	0	0	7	3	9	4
Other[e]	4	2	1	1	2	1	1	1	0	0	0	1	0
Total	72	67	35	16	5	2	2	28	4	15	4	11	5

[a] ECW = Pepper cultivar 'Early Calwonder'.
[b] 23-1 = Pepper cultivar 23-1-7.
[c] There was one nonpathogenic isolate that did not react with any of the *X. vesicatoria* antisera.
[d] Pathotype 1—pathogenic to tomato only; pathotype 2—pathogenic to tomato and ECW; and, pathotype 3—pathogenic to tomato, ECW, and 23-1.
[e] Includes *Physalis*, nightshade, and potato.

sera collected after a second course of immunization.

Double-diffusion tests were performed with sera having agglutination titers of 1,024 or 2,048. The 72 isolates were compared in double-diffusion tests against the seven antisera. The sonicated, and the whole-cell antigens, gave comparable number of bands and similar serological patterns in double-diffusion tests. There were 3-6 antigens in common among pathogenic isolates studied. This resulted in a high degree of serological relationship among all isolates that were typical of *X. vesicatoria* by their pathogenic reactions. Of the five nonpathogenic isolates, two each belonged to serotypes I and II, while the other was nonreactive with any *X. vesicatoria* serum.

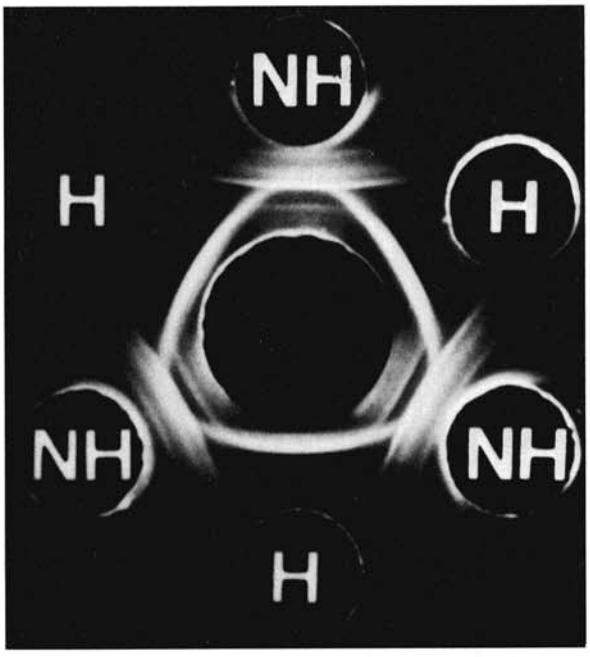

Fig. 1. The effect of heat treatment of antigens on the homologous serological reactions of *Xanthomonas vesicatoria* isolate 70-8 (Serotype I). Antiserum in center well and antigens in outer wells. NH = nonheated antigens; and H = heated antigens.

The nonheated antigens of isolates gave from 2-9 bands with different serum samples of *X. vesicatoria*. With heated antigens of isolates, there were 1-6 bands. Generally, heated antigens gave fewer bands with altered serological specificities from those of nonheated antigens. The heat treatment of antigens resulted in the formation of a band close to the antiserum well (referred to as "fast-moving band") and up to five other bands depending on the isolate (Fig. 1). There was correlation between the presence of 1-3 arclike bands close to antigen wells with nonheated antigens and the formation of this fast-moving band near the antiserum well with heated antigens (Fig. 2). When nonheated and heated antigens of isolates were compared in gel-diffusion reactions against antisera to isolates which were subsequently designated as type I, two kinds of precipitin patterns were evident. In the first pattern, seen with the majority of isolates, there was correlation between the presence of arclike bands of nonheated antigens and the formation of fast-moving bands with heated antigens. In the second pattern, the fast-moving band and arclike bands were faint or absent. Using the fast-moving band and the arclike peripheral bands formed against the above-mentioned sera, the isolates could be grouped into two types, I and II. The type I isolates formed arclike bands with type I sera and occasionally against type II, though the latter bands were less prominent than those of homologous type I reaction (Fig. 2). But type II isolates did not form arclike bands except against homologous - type sera, and such bands were distinguishable from the homologous type I bands. In addition, with type I isolates and type I sera the fast-moving band was relatively thick, whereas this band was thin in the homologous combination of type II. Of the seven isolates used for antiserum production, the pepper isolates (68-1 and 69-24), and tomato isolates (69-16, 70-8, and E3) belonged to the type I.

Isolates 70-7 and 69-13, from pepper and tomato, respectively, belonged to type II (Table 2). The gel-diffusion patterns of these isolates against the two types of sera are presented in Fig. 2. With the type I serum, it was possible to distinguish the two types of isolates, while type II serum did not distinguish the isolates clearly when heated or nonheated antigens were tested. Of the 72 isolates tested, 15 belonged to the type II, while the rest (except for one anomalous isolate) were of type I.

The cross-agglutination of the two types revealed specificity of each serotype. The agglutination titers of two sera belonging to each type are given in Table 2. The type I antigens agglutinated to a greater extent

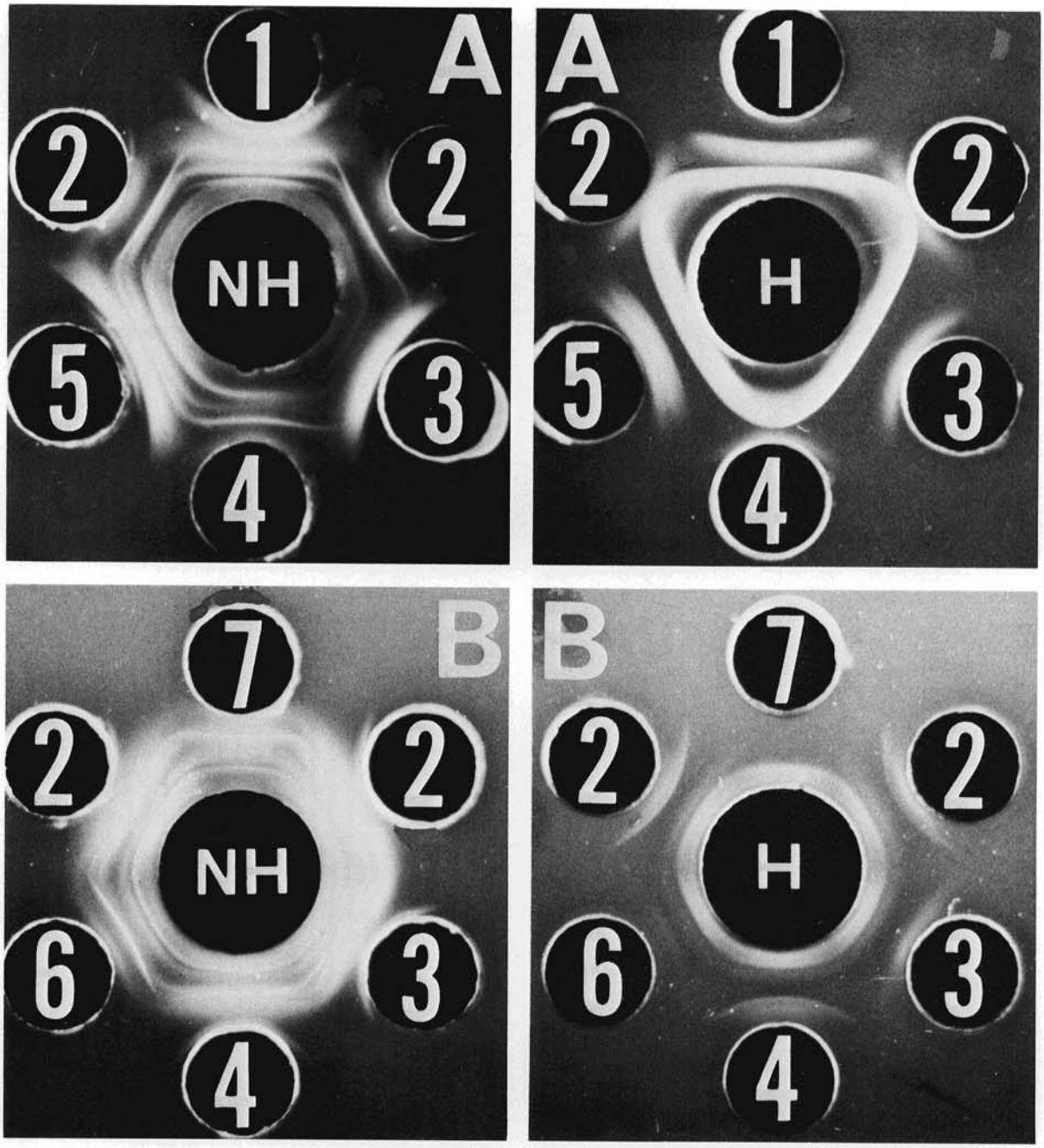

Fig. 2. Serological reactions of heated and nonheated antigens of *Xanthomonas vesicatoria* isolates belonging to two serotypes. Center wells in patterns contained antisera to isolate 69-16 (A) (Serotype I) and 69-13 (B) (Serotype II). Outer wells had nonheated (left) or heated (right) antigens of isolates. 1) 69-16; 3) 69-24; 5) 68-1; 6) 70-8; 7) E3 (Serotype I); 2) 69-13; and 4) 70-7 (Serotype II).

TABLE 2. Agglutinin titers of antisera of serotypes I and II of *Xanthomonas vesicatoria*

Antiserum to isolate	Serotype	Agglutinated with antigens of isolate[a] (host of origin)		Serotype	Titer
70-8	I	70-8	(T)	I	2,048
70-8	I	E3	(T)	I	2,048
70-8	I	69-13	(T)	II	4
70-8	I	70-7	(P)	II	8
69-24	I	69-24	(P)	I	1,024
69-24	I	69-16	(T)	I	1,024
69-24	I	69-13	(T)	II	4
69-24	I	70-7	(P)	II	4
70-7	II	70-7	(P)	II	512
70-7	II	69-13	(T)	II	256
70-7	II	69-24	(P)	I	8
70-7	II	70-8	(T)	I	16
69-13	II	69-13	(T)	II	512
69-13	II	70-7	(P)	II	256
69-13	II	69-24	(P)	I	8
69-13	II	70-8	(T)	I	8

[a] Agglutination was carried out in tubes, using fresh whole-cells of bacteria as antigens. T = tomato and P = pepper isolate.

in type I serum and vice versa.

Tests with heat-treated antigens of types I and II, fractionated by centrifugation into supernatant, washed pellets, and pooled washings of pellets revealed that the fast-moving bands were associated with supernatant fractions. Concentrated washings with warm saline (50 C) from nonheat-treated intact cells of type I isolates also gave the thick band specific for this type.

DISCUSSION.—The pathology and serology of isolates of *X. vesicatoria* tested were not always correlated (Table 1). Among isolates tested, two serotypes were designated on the bases of precipitin and agglutinin differences, the latter being more specific in distinguishing types. But the two types could not be associated with the host of origin of isolates or with such characteristics as virulence, host specificity, colony morphology, age of isolates in culture, streptomycin resistance, or starch hydrolysis. Our results disagree with those of earlier authors (13, 18) on serology of *X. vesicatoria*. This could be due to the fact that the isolates tested in each of these studies were different. The apparent strain differences reported earlier (13, 18) could have been due to a chance selection of different serotypic isolates from tomato and pepper. Though our study included fairly large numbers of isolates, it was by no means exhaustive. It seems likely that there are more serotypes or subtypes in *X. vesicatoria* whose identification will depend on further studies.

One of the five nonpathogenic isolates in this study did not belong to *X. vesicatoria* since it was nonreactive pathogenically and in gel-diffusion reactions. Though serology of four other isolates confirmed their relation to pathogenic *X. vesicatoria*, their inclusion in *X. vesicatoria* would be arbitrary since there is no adequate justification for doing so in the absence of pathogenicity.

Among the isolates of *X. vesicatoria* studied, there were no pathotypes specific for pepper. Cook & Stall (4) reported earlier that among the three pathotypes in Florida, there was a significant absence of pepper-specific isolates. However, the occurrence of pepper-specific isolates in nature is known (9). Presently, all pepper isolates were also pathogenic to tomato but some isolates were specific for tomato alone. The two serotypes were represented in all three pathotypes reported (4), and thus, identification of pathotypes with these serotypes was not possible. However, positive correlation between host specificity and serological specificity has been reported in other xanthomonads (11) and pseudomonads (16, 19).

Heat treatment of gram-negative bacteria to reveal heat-stable antigens has been useful (12, 14, 16, 19). Presently, the heat treatment of antigens has been valuable in revealing the specific reaction, but even nonheated antigens could aid in distinguishing serotypes of xanthomonads.

While the type I serum distinguished the heated antigens of types I and II readily, the type II serum did not. The relative thickness or thinness of fast-moving bands formed by heated type I or II antigens was a consistent characteristic in reactions with type I serum and thus was useful in distinguishing serotypes. The type II sera did not show this difference possibly because they possessed antibodies reactive with only a portion of the thick fast-moving bands of type I. This portion of antigenic determinants responsible for the thinner fast-moving band was common to both types, whereas the remaining antigenic determinants were specific for type I. This might explain the lack of distinction between fast-moving bands of types against type II serum while this was possible against type I serum.

Milner et al. (17) showed that fast-moving components could be formed from *Salmonella enteritidis* and *Escherichia coli* antigens, with or without a variety of treatments. These fast-moving components are serologically specific but nonantigenic. They were considered low molecular weight haptens arising from the complete O antigens that gave arclike bands close to antigen wells in gel-diffusion plates. There was relationship between the degree of hydrolysis of the complete antigen, the disappearance of the arclike bands, and the appearance of fast-moving bands in gel-diffusion plates (17). The soluble proteins of bacteria, when denatured, give rise to substances with altered serological specificity and mobility (2, 5). In our system, the fast-moving band of *X. vesicatoria* which is associated with cell-surface antigens might represent haptenic components of the somatic antigens or a mixture of polysaccharide and denatured protein antigens.

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