Differentiation of *Xanthomonas campestris* pv. *pruni* from Other Yellow-Pigmented Bacteria by the Refractive Quality of Bacterial Colonies on an Agar Medium

R. D. GITAITIS, Associate Professor, and J. D. HAMM, Research Technician II, Department of Plant Pathology, Coastal Plain Experiment Station, and P. F. BERTRAND, Associate Professor, Cooperative Extension Service, Department of Plant Pathology, Rural Development Center, University of Georgia, Tifton 31793-0748

ABSTRACT

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Epiphytic populations of *Xanthomonas campestris* pv. *pruni* were detected on peach leaves by spreading 0.1-ml aliquants from serial dilutions of leaf washes onto the surface of nutrient agar plates. After incubation at 30 C for 48–72 hr, petri plates were positioned 15 cm above a clear plastic template with horizontal black lines spaced 3 mm apart. The lined template was subilluminated by a fluorescent lamp light box. When the template was viewed through the bacterial colonies, various patterns were associated with different colony types. The clarity and refractive quality of colonies of *X. c.* pv. *pruni* created an undistorted image of discrete straight lines. Other yellow colonies distorted patterns or were opaque.

Bacterial spot of peach (*Prunus persica* (L.) Batsch) caused by *Xanthomonas campestris* pv. *pruni* (Smith) Dye sporadically causes heavy losses in south Georgia. The role of epiphytic populations in the epidemiology of bacterial plant pathogens is well documented (1,7–9). However, leaf surfaces can harbor a diverse microflora that can hinder detailed quantitative studies of a specific bacterial species. A selective isolation medium (XPSM) has been reported useful for detection of *X. c.* pv. *pruni* in natural situations (2). However, the plating efficiencies of XPSM and other selective media (6,10,11) described for *X. campestris* pathovars were too low for use when tested with *X. c.* pv. *pruni* strains from south Georgia. In a preliminary test (*unpublished*), a general purpose medium, nutrient agar, was used for detection and quantification of leaf-surface populations of *X. c.* pv. *pruni*. The manner by which colonies of that bacterium were distinguished from colonies of other yellow-pigmented bacteria on nutrient agar is addressed in this paper.

MATERIALS AND METHODS

Colonies of 10 strains of *X. c.* pv. *pruni*, originally isolated from diseased peach leaves in south Georgia, and three strains each of *X. c.* pv. *vesicatoria, X. c.* pv. *campestris, X. c.* pv. *malvacearum, X. c.* pv. *raphani*, and *X. c.* pv. *vignicola* isolated from tomato, cabbage, cotton, radish, and cowpea, respectively, were examined under routine laboratory conditions for their ability to act as a clear lens for viewing a lined template. Nutrient broth cultures were grown on a rotary shaker for 48 hr at 30 C for production of inoculum. Bacterial cells were harvested by centrifugation at 2,000 *g* for 15 min, and pellets were suspended in sterile phosphate buffer (0.01 M, pH 7.2) containing 0.85% NaCl (PBS). Bacterial suspensions were adjusted to 50% transmittance at a wavelength of 600 nm (corresponding with about 1×10^8 cfu per milliliter) with a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, NY). Bacterial suspensions were diluted serially (1:9), and 0.1 ml from selected dilutions was spread onto the surface of plates of nutrient agar. Plates then were incubated at 30 C for 48–72 hr. Plates resulting in 50–300 colonies were positioned approximately 15 cm above a subilluminated lined template; the height was adjusted until images in the colonies came into focus. Colonies were counted and scored for ability to allow for undistorted and unhindered views of the lines on the template. The lined template was subilluminated by a fluorescent lamp light box (20 J/sec) (SD 12-18/Glow-Box, Instruments for Research and Industry, Cheltenham, PA). The template consisted of horizontal black lines 1 mm wide and spaced 3 mm apart.

X. c. pv. *pruni* strain 84-22 was used in another study on the epidemiology of bacterial spot of peach to be presented at a later date. However, we used data from that study to evaluate the use of the lined template as a tool for identifying and counting colonies of *Xanthomonas* on dilution plates containing samples from natural habitats. Inoculum was prepared as described above. Ten peach trees (cv. Flordagold) were sprayed gently with inoculum using a hand-held, pressurized garden sprayer on 22 March 1985 and 19 March 1986. Leaf samples were collected immediately after inoculation and every 2 wk thereafter until 28 October in 1985 and 5 November in 1986. Twenty-four leaves were selected on each sample date and washed separately in test tubes containing 5 ml of sterile PBS. Chlorothalonil (0.1%; 0.5 g a.i./L, w/v) was added to the buffer to inhibit growth of fungal contaminants. Leaves were washed for 1 hr at 200 rpm on a reciprocating shaker. The resulting wash water was plated out in 1:9 dilutions onto plates of nutrient agar. The plates were incubated at 30 C for 48–72 hr and bacterial colonies were counted. Counting colonies was as outlined above, with plates held above the subilluminated template. Bacteria initially identified as *X. c.* pv. *pruni* strain 84-22 were further characterized by their morphology and key physiological tests (3), including aerobic and/or fermentative utilization of glucose, gelatin hydrolysis, Gram reaction, cellulolytic activity on carboxymethylcellulose, color, and starch hydrolysis, to verify their identity. Routinely, proteolytic (3) and cellulolytic (5) activities were the primary characteristics used to separate *X. c.* pv. *pruni* from other bacteria residing on peach leaves. Periodically, samples were tested for pathogenicity either by inoculations on peach foliage in the greenhouse or by testing for a hypersensitive reaction in tobacco.

The efficiency of the lined template for rapid identification of *X. c.* pv. *pruni* on nutrient agar was calculated by categorizing colonies by the patterns viewed through the colony. Colonies containing mostly discrete horizontal lines or lines only slightly curved were labeled as "suspect" colonies of *X. c.* pv. *pruni* strain 84-22. Colonies that were opaque or that showed other patterns, such as asterisks, convex curves, or lines pinched in the middle to form a lobe, were categorized as saprophytes. Colonies were then tested as above to verify whether they were *X. c.* pv. *pruni* or another bacterium. The tests were

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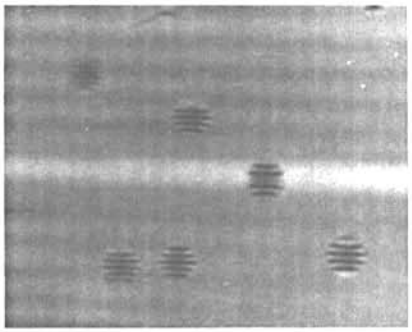

Fig. 1. Images of horizontal straight lines viewed through colonies of *Xanthomonas campestris* pv. *pruni* grown on nutrient agar. Nutrient agar plates were approximately 15 cm above a subilluminated plastic template marked with black horizontal lines.

Table 1. Identification of *Xanthomonas campestris* pv. *pruni* based on the refractive quality of bacterial colonies on nutrient agar[a]

Test number	Number of colonies tested	Colonies identified as nonpathogens		Colonies identified as *X. c.* pv. *pruni*		Colonies identified as possible *X. c.* pv. *pruni*	
		Number	Number correct[b]	Number	Number correct[b]	Number	Number correct
1	111	59	57	45	45	7	0
2	95	56	56	39	33	0	···
3	200	100	97	100	100	9	···
4	200	101	101	85	85	14	14
Total	606	316	311	269	263	21	14
Percent			98.4		97.8		66.7

[a] Based on viewing of horizontal lines on a plastic template, colonies of *X. c.* pv. *pruni* allowed appearance of discrete straight lines, whereas other bacterial colonies created distorted images or were opaque.

[b] Identities of bacteria were verified by physiological tests and pathogenicity (3,4).

repeated four times, with a total of 606 colonies categorized and tested.

RESULTS AND DISCUSSION

Horizontal straight lines with a minimum amount of curvature were observed in all colonies of 25 strains of the various pathovars of *X. campestris* tested when a lined template was viewed through individual colonies (Fig. 1). The technique was useful for the rapid identification and enumeration of xanthomonad colonies on dilution plates prepared from rinses of leaves sampled from commercial fields (Table 1). Based on the template alone, a decision of categorizing a colony as a saprophyte was correct 98.4% of the time. Bacteria categorized as "suspects" were identified as *X. c.* pv. *pruni* 97.7% of the time. Identifications were based on the presence of pale yellow pigmentation, proteolytic and cellulolytic activity but no starch hydrolysis, aerobic utilization of glucose, and cells being rod-shaped and gram-negative. When tested, all bacteria with these characteristics elicited a hypersensitive reaction in tobacco and caused typical bacterial spot symptoms on peach leaves in the greenhouse. Decisions were difficult to make in a small number (21 of 606) colonies, and these were categorized as suspect colonies with a lower degree of probability as to their actual identity; in these instances, the lined template method had only a 66.7% efficiency rating. With increased practice, however, the efficiency ratings could be improved even in these few instances.

Although there are more sophisticated and more thorough techniques available for the identification of bacteria, they may be too expensive or too time-consuming for the routine identification of large numbers of bacteria encountered in some epidemiologic studies. When dilution plates are prepared from samples from the field, a diverse microflora (in some cases even when selective media are used) may be present, which creates difficulty in quantifying the presence of the bacterium under study. Decisions on counting colonies of the correct organism often have to be made on colony characteristics, including color, elevation, form, margin type, opacity, size, and texture (4). Additional identifications (if any) then are made on representative examples. The lined template method provides additional information on the refractive quality and opacity of the bacterial colony that, when used along with other colony characteristics, increases the accuracy of counting colonies of *Xanthomonas* in epidemiologic studies. Follow-up identifications provided evidence of the accuracy of the technique. In addition, the method is inexpensive and requires little additional time and labor. Although the technique provides the researcher with additional confidence that the correct colony type is counted, it is still advisable to further characterize representative examples, as bacterial identifications should not be made solely on one characteristic. This is especially true until one is completely familiar with the opacity and the appearance of the colonies of the microflora associated with the particular plant host under study.

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