Two Strains of Pseudomonas eriobotryae Isolated from Loquat Cankers in California

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ABSTRACT

Two strains of *Pseudomonas eriobotryae* have been isolated from cankered loquat trees in California. Strain A produces brown pigment, does not liquefy gelatin, produces pectinase, and is phage-susceptible. Strain B produces no brown pigment, liquefies gelatin, does not produce pectinase, and is phage-resistant. Both strains, however, are serologically related, and both are pathogenic on loquat.

Additional key words: biochemical strains, bacterial canker.

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A stem canker disease caused by *Pseudomonas* sp. on loquat (*Eriobotrya japonica* [Thunb.] Lindl.) was recently reported in California (6). Symptoms were similar to the canker phase of a loquat disease in Japan caused by a bacterium, *P. eriobotryae* (Takimoto) Dows. (4, 10, 11, 15). A preliminary report (6) on the disease showed that the *Pseudomonas* sp. from cankered loquat trees is physiologically, pathogenically, and serologically distinct from *P. syringae*, and is serologically and pathogenically unrelated to *P. savastanoi*. The purpose of this study was to compare a number of isolates of *Pseudomonas* sp. from loquat cankers in California with a culture of *P. eriobotryae* from Japan.

MATERIALS AND METHODS.—Twenty-five cultures of *Pseudomonas* sp. isolated from infected loquat at different locations in Chico, Calif., were used in this study. The cultures were assigned State Laboratory accession numbers, and for these comparative studies the culture of *P. eriobotryae* from Japan was designated as SL 4083. The culture SL 4004 used in this study is described in an earlier report (6). All cultures were maintained on yeast dextrose calcium carbonate agar prior to the study.

Biochemical tests.—The following biochemical tests were used for characterizing the isolates: oxidase (14); starch hydrolysis (8); arginine dihydrolase (16); 2-ketogluconate (7); β-glucosidase (3); lipase (13); liquefaction of gelatin (8); and pectate gels (2).

Hypersensitive reaction on tobacco.—Leaves of tobacco (*Nicotiana tabacum* L.) were injected with a suspension of 10^8 to 10^9 bacterial cells/ml, and the inoculated plants maintained on the greenhouse bench (5). Positive reactions on tobacco leaves were recorded 24 hr after inoculation.

Phage reaction.—The method used to isolate bacteriophage involved enrichment culture, and was essentially like that described by Crosse & Hingorani (1). About 50 g of soil collected beneath cankered trees were dispensed into 250-ml Erlenmeyer flasks containing 50 ml of 48-hr-old bacterial culture SL 4004. The mixture was incubated at 25 C on a rotary shaker for 48 hr, clarified with filter paper, and centrifuged at 3,000 *g* for 30 min. About 30 ml of supernatant were transferred to a separatory funnel and shaken vigorously with 0.5 ml of chloroform to kill bacteria. After separation of the chloroform and the aqueous phase, 1 ml of the aqueous fraction was withdrawn, mixed with 5 ml of melted phage agar (0.75%) containing 1.0% peptone and 0.5% NaCl, and layered over a standard nutrient agar (Difco) in which SL 4004 was suspended as the propagating culture. The plates were dried at 37 C, and incubated at 25 C. Plaques were observed after 24 hr, and varied with different soil samples from a few to several hundred/plate. Some material from isolated lytic spots was suspended in 18-hr broth cultures of SL 4004 to increase the phages in phage medium from which agar was omitted. We purified the phages by streaking a drop of phage suspension with an L-shaped glass rod on the dried surface of phage agar sown with 18-hr-old cultures of SL 4004. The discrete plaques were cut from agar and again propagated in the broth culture. The purification process was repeated 2 to 3 times. To test phage reaction, very turbid bacterial suspensions of 16-hr-old cultures of each isolate were added into the melted phage agar, and the mixtures were then poured over 2% nutrient agar and spotted with a purified phage preparation. Phage reactions that differed in amounts of lysis were recorded at 24 hr after inoculation.

Pathogenicity tests.—All cultures of the pseudomonads isolated from loquat cankers were first tested for oxidase. Oxidase-negative cultures were tested for pathogenicity. Stems of loquat seedlings ca. 2 ft high were wound-inoculated at various locations with 48-hr-old cultures on Pseudomonas agar F (Difco). Inoculated loquat seedlings were covered loosely with plastic bags and kept in a moist chamber for 4 to 5 days, after which the coverings were removed and the plants maintained on a greenhouse bench. Readings for pathogenicity were made 5 weeks after inoculation.

Serological tests.—Agglutination tests were run on SL 4004, SL 4083, and SL 4104, because they showed some distinct biochemical differences in

TABLE 1. Differences in biochemical and phage reactions of isolates of *Pseudomonas eriobotryae*

Strain	Isolate no. (SL)	Gelatin	Brown pigment	Pectate gel at pH 5.5	Pectate gel at pH 7.0	Reaction to phage[a]
A	4004	–	+	+	+	VS
	4004M	–	+	+	+	I
	4008	–	+	+	+	VS
	4018	–	+	+	+	VS
	4043	–	+	+	+	VS
	4048	–	+	+	+	S
	4058	–	+	+	+	VS
	4059	–	+	+	+	VS
	4060	–	+	+	+	VS
	4061	–	+	+	+	VS
	4062	–	+	+	+	VS
	4063	–	+	+	+	VS
	4072	–	+	+	+	VS
	4095	–	+	+	–	VS
	4096	–	+	+	+	VS
	4097	–	+	+	+	VS
	4098	–	+	+	+	VS
	4099	–	+	+	+	VS
	4100	–	+	+	+	VS
	4101	–	+	+	–	VS
	4102	–	+	+	+	VS
	4103	–	+	+	+	VS
	4105	–	+	+	–	VS
	4106	–	+	+	+	VS
A (?)	4083[b]	–	–	+	+	VS
B	4104	+	–	–	–	R

[a] VS = very susceptible; S = susceptible; R = resistant; I = immune to phage lysis.
[b] Culture obtained from Japan.

brown pigment, in pectinase production, and in liquefaction of gelatin. Twofold dilution sera starting at 10^{-1} of its original strength in a series of 10 X 75-mm test tubes were prepared, using as diluent 0.85% sterile saline solution buffered at pH 6.5 with 0.01 M phosphate. One-half ml of a suspension (phosphate-buffered saline) of cells from 16-hr-old cultures grown on Pseudomonas agar F was added to the same amount of dilution sera. The antiserum was the same as that used for investigations previously (6) reported. Both normal serum and saline were used as controls for each isolate. Readings were made after 24 hr at room temperature. Agar-gel (0.85% Ionagar and 0.05% sodium azide) diffusion tests were performed in the pattern of six peripheral antigen wells located 4 mm from the central antiserum well. To give a strong antigen-antibody reaction, the turbid antigens in saline and undiluted antiserum were used. The plates were kept in a moist chamber at room temperature (22-25 C) for 48 hr. The results were recorded on the 3rd day.

RESULTS.—Results of differential tests are shown in Table 1.

Biochemical tests.—All isolates were unable to hydrolyze starch or to produce oxidase and arginine dihydrolase within 7 days, or 2-ketogluconate within 10 days. They all produced β-glucosidase and lipase, reacting slowly on Tween 80 (polyoxyethylene sorbitan monooleate) agar within 10 days. Isolate SL 4104 strongly liquefied gelatin within 24 hr; all others were negative at the end of 5 weeks.

On pectate gel at pH 5.5, SL 4104 was the only isolate that did not produce pitting. At pH 7.0, all produced pitting except SL 4095, SL 4101, SL 4104, and SL 4105. At pH 8.5, none pitted the medium. All pitting developed within 24 hr; cultures were held for 2 weeks.

Isolates SL 4083 and SL 4104 did not produce a visible pigment on Tween 80, Pseudomonas F, or nutrient agars within several days. All others produced a diffusible brown pigment.

Hypersensitive reaction.—All isolates elicited strong hypersensitive reactions within 24 hr in tobacco leaves injected with suspensions containing ca. 10^9 cells/ml.

Phage reactions.—Isolate SL 4104 which developed a very faint lysis zone was resistant to phage lysis within 48 hr (Fig. 1). All other isolates which developed partially or completely cleared zones were susceptible or extremely susceptible within 24 hr. However, a mutant colony in culture SL 4004, subsequently propagated as SL 4004M, remained completely immune to lysis, yet unchanged in all other characteristics. All phage-susceptible isolates developed resistance to lysis after 72 hr.

Pathogenicity.—All isolates obtained from loquat cankers produced characteristic stem cankers within 5 weeks after inoculation of loquat seedlings.

Serological reactions.—Antiserum of SL 4004 agglutinated all isolates, including the phage-resistant

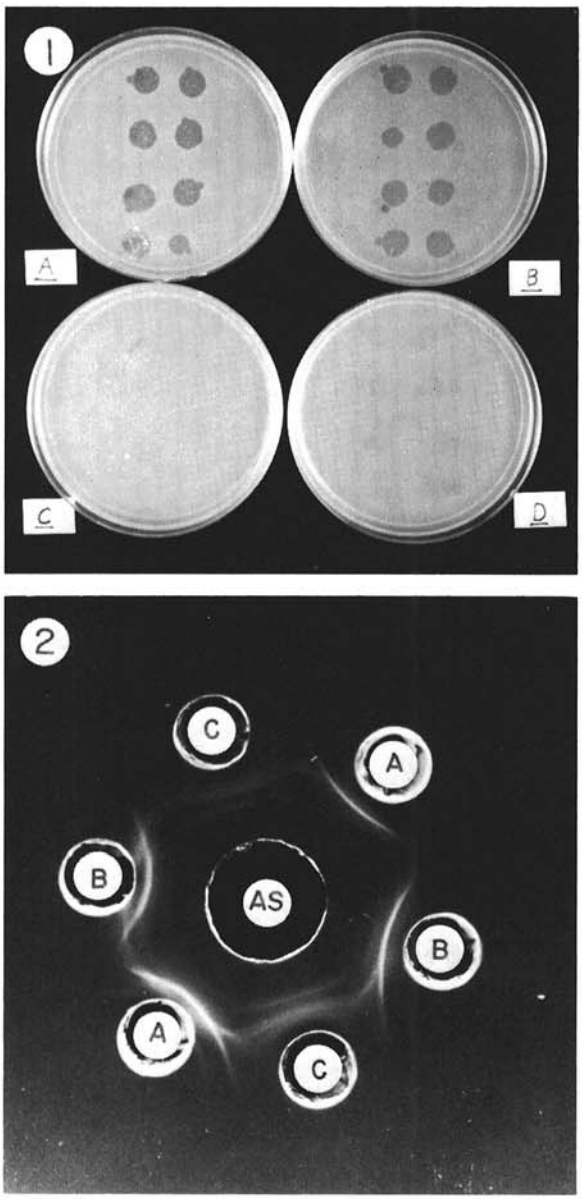

Fig. 1-2. 1) Bacteriophage lysis on the culture plates of *Pseudomonas eriobotryae*: A = isolate SL 4004; B = isolate SL 4083; C = mutant SL 4004M; and D = isolate SL 4104 at 72 hr. **2)** Agar-gel diffusion patterns of isolates of *P. eriobotryae*: AS = SL 4004 antiserum; A = antigen SL 4004; B = antigen SL 4104; and C = antigen SL 4083.

mutant SL 4004M. Representative titers using antiserum of SL 4004 against SL 4004, SL 4004M, SL 4083, and SL 4104 were 1/1,280, 1/1,280, 1/640, and 1/640, respectively. With normal serum, no agglutination was detected with any isolate. In gel diffusion tests, using the same antiserum and representative isolates SL 4004, SL 4083, and SL 4104, two common connecting lines formed and a third continuous line formed with SL 4004 and SL 4104 (Fig. 2). This indicates serological identity between SL 4004 and SL 4104 and serological relationship with SL 4083.

DISCUSSION.—The 25 isolates of *Pseudomonas* sp. obtained from loquat cankers in California are all greenish fluorescent pseudomonads which are negative for production of oxidase and arginine dihydrolase (9, 12) and positive for hypersensitive reaction on tobacco leaves (5). These characteristics fitted pathogenic pseudomonads described in previous reports (9, 12). Results of other biochemical reactions and serological and bacteriophage lysis tests indicate that the characteristics of California cultures, excluding SL 4104, are similar to those of the culture (SL 4083) of *P. eriobotryae* from Japan. Also, all California cultures and SL 4083 were pathogenic on loquat. Therefore, the pathogenic pseudomonads isolated from loquat canker are considered to be *P. eriobotryae*.

Distinct differences in phage reaction, formation of brown pigment, liquefaction of gelatin, and production of pectinase on pectate gels indicate that the cultures fall into two groups and consequently are considered strains, designated A and B. Viewed in this context, most of the isolates are strain A, the prevailing wild-type strain in the Chico area. The single isolate SL 4104 is strain B, a minor strain. Isolate SL 4083 is intermediate, but probably should be included in strain A. Moreover, serological studies, including agar-gel diffusion and agglutination, indicate that strains A and B are serologically (6) identical.

Isolate SL 4083 from Japan, in spite of its lack of brown pigment production, is closely related to strain A. At present, no isolates with characters identical to SL 4083 lack pigment, and both cannot be considered as being the same strain. Okabe (11) reported three strains of *P. eriobotryae* in Japan with differences in formation of brown pigment, and the utilization of sucrose and galactose as carbon sources. He also indicated that the strain which did not produce brown pigment was considered a naturally occurring variant of the brown, pigment-producing strain. Consequently, strain A found in California is identical to a wild-type strain 0-1 in Japan. We consider that strain B shows different enough characteristics not to be placed in any of three strains previously described (11).

Okabe (11) and Muko (10) reported that isolates of *P. eriobotryae* from loquat cankers can liquefy gelatin. This is in contrast to our finding that all cultures of strain A and SL 4083 are unable to liquefy gelatin; however, strain B liquefies gelatin vigorously.

Although pathogenicity and serological tests revealed that strains A and B apparently are identical, inoculation of a wide range of host varieties may reveal differences between these strains.

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