Characterization and Serological Comparisons of Bacteria of the Genus Erwinia Associated with Discolored Alfalfa Roots

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ABSTRACT

Several isolates of *Erwinia* spp. bacteria originally isolated from discolored roots of alfalfa (*Medicago sativa* L.), were subjected to selected physiological and nutritional tests. These bacteria were gram-negative, nonspore-forming, encapsulated rods with peritrichous flagella. They were oxidase-negative, unable to utilize lactose and rhamnose, lacked pectinase, and metabolized glucose fermentatively. These characters suggest that the alfalfa isolates belong to the 'amylovora' group of the genus. The comparison of certain physiological and serological characters of six *Erwinia* isolates from alfalfa with three known phytopathogenic *Erwinia* spp. showed that the former isolates were distinct from the other described phytopathogenic species of the 'amylovora' group. We propose the name *E. amylovora* var. *alfalfae*. A serological comparison showed that four of the six isolates tested produced a reaction of identity in gel-diffusion tests.

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A root disease complex of alfalfa (*Medicago sativa* L.) involving *Fusarium* spp. and two groups of gram-negative bacteria were observed in areas of eastern Pennsylvania. These bacteria were either Pseudomonads or fit the description for the genus *Erwinia*.

The observations on isolation, pathogenicity, and characterization of fluorescent Pseudomonads involved in this disease complex have been reported (19). A review of the literature failed to detect any reference to *Erwinia* sp. occurring in the discolored roots of alfalfa. This report is a summary of our studies to determine the identity and pathogenicity of these bacteria. Work showing the role of these two groups of bacteria in the development of wilt and root-rot of alfalfa will be published separately elsewhere.

MATERIALS AND METHODS.—The methods of isolation and inoculation, as well as different physiological and nutritional tests described for the characterization of fluorescent pseudomonads from alfalfa (19), were used to characterize the isolates of *Erwinia* recovered from the same source. These isolates will be referred to as the "alfalfa Erwinias." In addition to these tests, acid production from xylose, sorbitol, and rhamnose after 3, 7, 14, and 21 days incubation was determined using Dye's medium C (3). The carbon-source compounds (0.5%, w/v) were added asceptically after membrane sterilization. Pigment production was determined after 2, 3, and 10 days incubation on King's A medium (12), Miller's selective medium (17), yeast-dextrose calcium carbonate agar (20), beef-lactose agar (BLA), and a modified Eosin methylene blue (EMB) agar (20).

A serological comparison of Erwinia isolates.—Bacteria grown on beef-lactose agar (BLA) for 4-5 days at 27 C, were suspended in sterile formalin-saline (10 ml of 40% formaldehyde/liter of saline((10). After storage at 27 C for 48 h the bacterial cells were sedimented by centrifugation, washed twice with sterile formalin-saline, and resuspended in formalin-saline. Sterility of the antigen preparation was checked by streaking the suspension on BLA plates. The concn of bacterial cells was adjusted to 9×10^8 cells/ml (McFarland scale tube 3) (1) for the inject antigen and the agglutination test. Two types of antigens were prepared for the gel-diffusion test. Bacterial cells from 6-day-old colonies on BLA plates were suspended in saline, centrifuged and washed twice with saline, then adjusted to 9×10^8 cells/ml. This suspension was divided into two lots. One lot was

sonicated until it became opalescent (20-25 min at a setting of seven, on a Branson Model IS-75 sonicator) and the second was heated in boiling water for 2 h. The antigens were stored at 4 C until needed.

Antisera were produced in healthy female New Zealand white rabbits weighing 2.0-2.5 kg. Two rabbits were used for each bacterial isolate. Pre-immunization serum was collected. Antisera were produced during August-September, 1971, against four isolates of the alfalfa Erwinias (E-1, E-2, E-3, and E-4). Isolates E-5 and E-6 were obtained in the Spring of 1972. These two isolates, and three known species of *Erwinia* [*E. amylovora* (apple isolate), *E. amylovora* var. *rubrifaciens*, and *E. caratovora* var. *rhapontici*] were included in the tube agglutination and gel-diffusion tests. The initial injection consisted of 0.25 ml of antigen in the marginal ear vein and 2.0 ml of antigen, emulsified with 2.0 ml of Freund's incomplete adjuvant, in the hip muscle. Subsequent injections in the marginal ear vein were increased by 0.25 ml, at three-day intervals, until 1.50 ml was reached. Five days after the last injection, rabbits were bled from the marginal ear vein. After 1 wk of rest a booster dose of 1.75 ml antigen was injected into the marginal ear vein and 2.0 ml antigen emulsified with 2.0 ml adjuvant into the hip muscle. Four days later, final bleeding was done by cardiac puncture. Serum from each rabbit was stored frozen, without preservative, until needed.

Antibody titer of the different antisera was determined by the extinction dilution method (2). Agglutination tests for homologous and heterologous titer determinations were done in tubes (13 × 100 mm) incubated at 37 C in a water bath for 2 h. The tubes were read twice for agglutination against the control; first, immediately after incubation in the water bath, and once again after overnight storage in the refrigerator.

The Ouchterlony agar gel-diffusion method (18) was employed to determine serological relationships among the various antigens and four antisera of the Erwinias. Gel-diffusion tests were made in 90-mm diam plastic petri dishes containing 15-20 ml of 1.0% Epiagar (Colab Lab, Inc.) in 0.01 M potassium phosphate buffer-saline (1.18 g K_2HPO_4, 0.34 g KH_2PO_4/liter of saline, pH 7.2). After sterilization of the medium, but before pouring plates, 0.4 ml/liter of liquid phenol (80%, v/v) was added as preservative. The plates were freshly prepared and allowed to dry 24 h before use. A circular pattern, with six outer wells (7 mm in diam, 10 mm apart and 10 mm from the central well) surrounding a central well (10 mm in diam), was cut with a Feinberg agar-gel cutter (Colab Lab, Inc.). Antigen and antisera were prepared to the desired concn by dilution with sterile physiological saline, placed in wells in various combinations and incubated in 4 C for 15 days. Wells were refilled once or twice with the antigens and antisera.

RESULTS.—*Isolations.*—Of 61 infected plants tested, the Erwinias were present in 15 plants, of which 12 samples also had fluorescent pseudomonads and *Fusarium* spp. The Erwinias were recovered from the other three infected plants with fluorescent pseudomonads. Of the 15 samples of apparently healthy plants, Erwinias were present along with *Fusarium* and fluorescent pseudomonads in two plants, and with *Fusarium* in one of the plants.

Pathogenicity tests.—All of the isolates of *Erwinia* tested produced a slight to moderate necrosis of roots within 4 wk after inoculation in a preliminary inoculation test. In greenhouse inoculation tests, the Erwinia isolates caused a moderate yellowing of leaves followed by death of the plant. However, only isolates E-3 and E-6 caused severe necrosis of roots of wilted plants. The control plants had dark green leaves and were growing vigorously.

Morphological characterization of Erwinias.—The *Erwinia* isolates were gram-negative, nonspore-forming rods with slightly pointed ends. All produced capsules. Active motility was seen in hanging drop preparations with the phase contrast microscope. Electron micrographs revealed peritrichous flagellation, with the number of flagella ranging from 4-13.

Physiological characterization tests.—The *Erwinia* spp. isolates did not produce levan. Colonies produced by these isolates were medium in size, white, convex, and smooth. No oxidase was detected on nutrient agar and King's B medium. The isolates did not produce any arginine dihydrolase. The isolates tested were not capable of hydrolyzing starch in 10 days after inoculation. All of the Erwinias failed to produce any pitting on Hildebrand's media A, B, and C (10), indicating that no pectolytic enzymes were produced. None of the isolates were able to cause softening of potato slices. They also did not incite a positive hypersensitive reaction when injected into tobacco leaves. All the isolates of Erwinias tested failed to produce indole, even after 11 days of incubation. The Erwinias gave a negative methyl red reaction in glucose-phosphate-peptone water; in Dye's (3) second medium ($NH_4H_2PO_4$, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.5 g; yeast extract, 1.0 g; glucose, 10 g; distilled H_2O, 1.0 liter; pH 6.8), however, all gave a positive methyl red test after 2 days of incubation.

All isolates of Erwinias were strong gelatin liquefiers. Complete liquefaction of medium in tubes by these isolates was accomplished within 14 days after inoculation. In plates of gelatin, the isolates produced a clear zone of 10-12 and 20-25 mm around the colony in 3 and 7 days after inoculation, indicating rapid hydrolysis of gelatin. On the Tween-80 medium, the Erwinias produced an opaque halo of about 5-6 and 15-20 mm, around the bacterial growth in 3 and 7 days, respectively, which is a positive indication of lipase production. The Erwinias gave a positive catalase reaction. Hydrogen sulphide was detected within 2 days of incubation. The Erwinias gave a positive nitrate reduction at 1 and 5 days after inoculation. In glucose-phosphate-peptone water, 4 days after inoculation, the Erwinias gave a positive V-P reaction whereas negative reaction was obtained in Dye's second medium. The Erwinias grew throughout the medium, an indication of facultative anaerobic activity.

—1) Action on carbohydrates.—The Erwinias produced acid from sucrose within 3 days of incubation. All the isolates of Erwinia tested produced acid from xylose and sorbitol. However, acid production in sorbitol was much faster than in xylose. No isolate was able to produce acid from rhamnose, even after 20 days of incubation. The Erwinias failed to produce either acid or gas from lactose. Rapid acid production from glucose, without any trace of gas production, was observed 4 days

after inoculation; and by the sixth day, the entire liquid medium turned yellow. In a test to determine the type of glucose metabolism, the Erwinia tested produced an acid reaction throughout the medium in both tubes, suggesting the presence of a fermentative metabolism of glucose.

—2) Pigment production.—All the alfalfa Erwinia isolates except E-4 produced a light pink diffusible pigment on King's A medium and on BLA within 7 days of incubation. Only two isolates (E-5 and E-6) produced a light pink pigment which accumulated at the butt of YDC agar slopes in 7 days. This pink pigment was very light compared to a red diffusible pigment produced in this medium by *E. amylovora* var. *rubrifaciens*. After 24-48 h on Miller's medium (18), which is selective for Erwinias, colonies of all isolates as well as those of the three known *Erwinia* spp. turned orange. The medium also turned orange. Colonies and the medium of all of the alfalfa Erwinias under study and two of the three known species of Erwinia (*E. amylovora* and *E. caratovora* var. *rhapontici*), however, soon turned a light-bluish-green in color. Only in the case of *E. amylovora* var. *rubrifaciens* did the orange color of the colonies and the medium persist for more than 15 days. All isolates of the alfalfa Erwinias and the three known *Erwinia* spp. were streaked on a modified EMB agar plates. On EMB agar I (with glucose instead of lactose), all the isolates of the Erwinias under study developed colonies that were deeply stained (pink-red), and showed a green metallic sheen in reflected light. *E. amylovora* produced deep orange-red fluidal colonies without metallic sheen, *E. amylovora* var. *rubrifaciens* produced metallic sheen without deep pigmentation of growth and *E. caratovora* var. *rhapontici* developed prominent metallic sheen with little

TABLE 1. Homologous and heterologous titers of six *Erwinia* isolates (E-1 to E-6) from discolored alfalfa roots, and three plant-pathogenic *Erwinia* spp.

Antigen	Antisera against antigens of:			
	E-1	E-2	E-3	E-4
E-1	2,560[a] (++[b])	1,280 (+)	640 (+)	5,120 (++)
E-2	2,560 (+)	1,280 (+)	640 (+)	5,120 (++)
E-3	2,560 (++)	1,280 (++)	1,280 (++)	5,120 (++)
E-4	2,560 (++)	1,280 (+)	1,280 (+)	5,120 (++)
E-5	1,280 (++)	1,280 (+)	640 (++)	5,120 (++)
E-6	2,560 (+++)	1,280 (+)	1,280 (+)	2,560 (+)
E. amylovora var. *rubrifaciens*	0	0	0	0
E. carotovora var. *rhapontici*	0	0	20 (+++)	20 (+++)
E. amylovora	20 (+)	0	0	0

[a]Number represents the denominator of the greatest dilution of serum clearly showing agglutination.
[b]Plus marks in parentheses indicate the amount of aggregation of a bacterial antigen by the indicated antisera: + = slight but positive aggregation; ++ = moderate aggregation; +++ = moderately heavy; ++++ = heavy aggregation.

TABLE 2. Number of precipitin bands produced in gel-diffusion tests from the interaction of four antisera and two types of antigens[a]

Antigens	Antisera against antigens of:			
	E-1	E-2	E-3	E-4
A. *Sonicated cells*				
E-1	s-1, d-1, f-1	0	s-1, d-1	0
E-2	0	d-1, f-1	0	d-2
E-3	s-1, d-5, f-1	0	s-1, d-2, f-1	0
E-4	0	0	0	s-1, d-2
E-5	s-1, d-2, f-2	0	s-1, d-3, f-2	d-2
E-6	s-1, d-2, f-1	f-1	s-1, d-3, f-1	d-2
E. amylovora	d-2	f-1	f-1	0
E. amylovora var. *rubrifaciens*	0	0	0	0
E. carotovora var. *rhapontici*	d-1, f-1	f-1	f-1	0
B. *Boiled cells*				
E-1	d-2	0	d-1, f-1	0
E-2	0	d-1	0	0
E-3	d-2	0	d-1	0
E-4	0	0	f-1	d-2
E-5	d-1, f-2	d-1	d-1	0
E-6	d-2, f-3	d-1	d-1, f-1	0
E. amylovora	0	0	0	0
E. rubrifaciens	0	0	0	0
E. rhapontici	0	0	f-1	0

[a]Results of gel-diffusion tests, 15 days after first filling of wells and at 4 C incubation in refrigerator.
[b]s = strong, prominent band; d = distinct, clear band; f = faint, thin band.

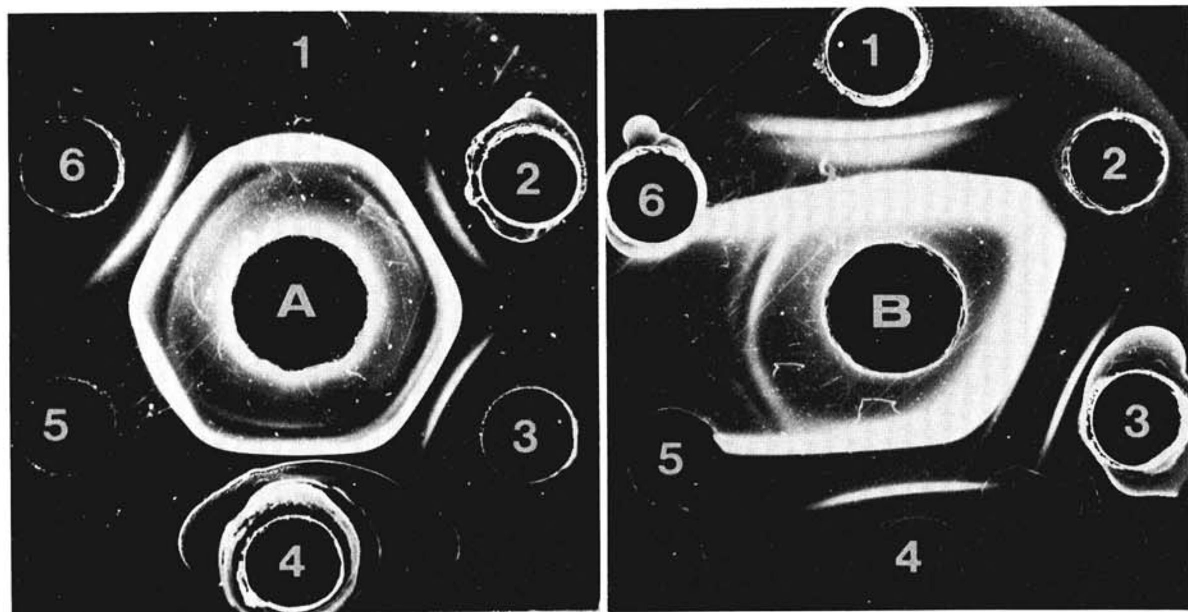

Fig. 1. Precipitin band pattern formed with antiserum prepared against *Erwinia* spp. (E-1) from discolored alfalfa roots and homologous antigens and antigens from selected known plant-pathogenic *Erwinia* spp. (The antigens were sonicated). Center well (A) contained antiserum against the following E-1 antigens in outer wells: 1) E-1; 2) E-5; 3) E-3; 4) E-6; 5) E-1; and 6) E-3. Center well (B) contained antiserum against E-1 antigens in outer wells: 1) E-5; 2) *E. amylovora* var. *rubrifaciens*; 3) E-6; 4) E-1; 5) *E. amylovora*; 6) *E. carotovora* var. *rhapontici*.

pigmentation of colonies. On EMB II [with ammonium sulphate (10 g/liter) and glycerol (10 ml/liter) instead of peptone and glucose, respectively] all the test isolates produced a metallic sheen without deep pigmentation of bacterial growth, whereas *E. amylovora* did not develop either a metallic sheen or a deep pigmentation. *E. caratovora* var. *rhapontici* developed a prominent metallic sheen without deep pigmentation. Only traces of the metallic sheen, without development of deep pigmentation, was produced by *E. amylovora* var. *rubrifaciens*.

—3) Nutritional tests.—The organic compounds tested as substrates were: cellobiose, salicin, sorbitol, glycine, β-alanine, L-valine, L-proline. All of the Erwinias were able to utilize each of these substrates except L-valine.

A serological comparison of Erwinia isolates.—Tube agglutination and gel-diffusion tests, completed with normal serum to determine interaction with test antigens, were negative. The homologous titer of the antisera, collected after the final booster antigen injection ranged from 640 to 5,120. Antigens of the six isolates of the alfalfa Erwinias under study also produced a high titer in heterologous cross-reactions (Table 1). *E. amylovora* var. *rubrifaciens* did not agglutinate with any of the antisera. *E. amylovora* and *E. caratovora* var. *rhapontici* reacted at a very low titer with E-1 and E-2 antisera, respectively (Table 1).

Gel-diffusion tests with sonicated antigens produced a greater number of precipitin bands than those with boiled-cell antigens (Table 2). At least one strong band showing identity was produced by antigens of E-1, E-3, E-5, and E-6 with E-1 antiserum (Fig. 1-A). Antigens of E-2, E-4, and *E. amylovora* var. *rubrifaciens* did not produce any band with E-1 antiserum. However, bands produced by *E. amylovora* and *E. caratovora* var. *rhapontici* with E-1 antiserum did not shown reaction of identity (Fig. 1-B). Similar results were obtained with E-3 antiserum. In the case of E-2 antiserum, antigens of E-2, E-6, *E. amylovora* and *E. caratovora* var. *rhapontici* reacted each with one faint band showing identity. With E-4 antiserum, antigens of E-5 and E-6 each produced two unrelated bands, whereas antigens of E-1, E-3, *E. amylovora*, *E. amylovora* var. *rubrifaciens*, and *E. caratovora* var. *rhapontici* did not produce any bands.

DISCUSSION.—The genus *Erwinia*, as defined by Dye (6), consists of a group of usually peritrichously flagellated, gram-negative, nonspore-forming rods. They are catalase-positive, oxidase-negative, do not hydrolyse starch, are facultative anaerobes, and many cause plant diseases. The isolates from the discolored alfalfa roots have these characteristics. Based on his study of the different species in the genus *Erwinia*, Dye (3, 4, 5, 6) recently divided members of this genus into three major groups: viz. (i) the *amylovora* group; (ii) the *caratovora* group; and (iii) the *herbicola* group. The similarity between the biochemical characters of the organisms in the 'amylovora' and 'carotovora' group is used to justify their inclusion into one genus (4, 14, 15). However, three major characteristics separate them. The 'amylovora' group lacks pectinase (or the ability to cause soft-rot)., lactose utilization and nitrate reduction (4, 11). With few exceptions, these characteristics are uniform within the two groups regardless of species designation. The alfalfa Erwinias tested lack pectinase, as well as pectolytic enzymes. They are unable to utilize lactose and rhamnose,

TABLE 3. Comparison of certain physiological characters of *Erwinia* isolates from alfalfa with three selected plant-pathogenic *Erwinia* spp.

Characterization tests	Erwinia isolates from alfalfa roots	*E. amylovora*[a]	*E. amylovora* var. *rubrifaciens*	*E. carotovora* var. *rhapontici*[c]
Pectate liquefaction	-	-	-	-
Nitrate reduction	+	-	-	+
Gelatin liquefaction	+	+	-	-
Catalase reaction	+	+	+	-
Gas from glucose	-	-	-	-
H_2S production	+	-	-	-
Production of acid from:				
Xylose	+	+	-	+
Rhamnose	-	-	-	+
Sorbitol	+	+	+	+
Lactose	-	-	-	-
Hypersensitivity	-	+	0	0
Diffusible pigment on:				
Yeast extract dextrose calcium carbonate agar	-	-	Red	Red-pink
King's A medium	Light pink[d]	-	Mod. pink	Light pink
Beef lactose agar	Light pink	-	-	Light pink

[a]Data from Dye (3), Elliott (7).
[b]Data from Dye (3), Wilson et al. (20).
[c]Data from Dye (4), Wilson et al. (20), Metcalf (16).
[d]Pigmentation after 7 days of incubation at 27 C.

but are capable of producing acid from glucose, xylose, sorbitol, and sucrose. On account of this, the six isolates under study came very close to the 'amylovora' group. However, they gave a positive nitrate reduction test.

The production of a water-soluble light-pink pigment on King's A and BLA by five of the six isolates under study is a relatively stable characteristic. Other species of *Erwinia* known to produce red or pink pigment are *E. amylovora* var. *rubrifaciens* which produces a red pigment on YDC (20), and *E. caratovora* var. *rhapontici* which produces a pink pigment on autoclaved potato (16). A comparison of several selected physiological tests of the *Erwinia* isolates from alfalfa with the above *Erwinia* spp. show that the alfalfa isolates differ in certain physiological tests (Table 3). A serological comparison of *E. amylovora*, *E. amylovora* var. *rubrifaciens* and *E. caratovora* var. *rhapontici* with the alfalfa Erwinias showed that only the alfalfa isolates reacted to a high titer in heterologous cross-reactions. These high titers indicate that these isolates are closely related (8, 13). Absence of, or very low, agglutination titer of the three known *Erwinia* spp. tested shows that they are serologically unrelated to the alfalfa Erwinias. In the gel-diffusion tests, with E-1 and E-3 antiserum, four of the six alfalfa isolates showed a reaction of identity with a major antigenic component. Isolate E-2, E-6, *E. amylovora*, and *E. caratovora rhapontici* reacted with one faint band with E-2 antiserum, whereas with the E-4 antiserum, only E-5 and E-6 shared two unrelated antigenic components. Results of gel-diffusion tests also show that the three known *Erwinia* spp. tested are serologically unrelated to the alfalfa isolates. However, the differences observed in the reactions of E-2 and E-4 isolates in tube-agglutination and gel-diffusion tests could be due to the modification of external antigens of these isolates by ultrasonic oscillation. Similar phenomena have been observed with *Rhizobium meliloti* in the work reported by Gibbins (9).

The data summarized and presented in Table 3 show that even though the alfalfa Erwinias differ from *E. amylovora* in their ability to reduce nitrate, produce H_2S, produce pigment, and tobacco leaf hypersensitivity, they resemble very closely *E. amylovora* in many other characters. In the light of the observations regarding major differences and similarities between *E. amylovora* and the alfalfa Erwinias, it is felt that these isolates are distinct from the other described species of the 'amylovora' group. Since these isolates are frequently associated with discolored alfalfa roots and are significantly different from known Erwinia species, it is justified to consider these alfalfa isolates as a new variety of *E. amylovora*. We therefore propose to call the alfalfa Erwinias "*E. amylovora* var. *alfalfae*," with isolate E-3 as the type culture. This conclusion is consistent with the currently accepted thinking on the taxonomy of genus *Erwinia* (3, 4, 5, 6).

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