# Association of *Serratia marcescens* with Crown Rot of Alfalfa in Pennsylvania

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## ABSTRACT

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Comparative investigations of the physiological and biochemical properties of strains of a bacterium isolated from alfalfa and designated *Erwinia amylovora* var. *alfalfae* and a test isolate of *Serratia marcescens* showed that they differ by production of pigments with different solubility properties and absorption spectra. Otherwise, the results of the biochemical tests for the two strains were similar. The guanine-plus-cytosine content of the DNA from the alfalfa strains ranged from 56.6 to 57.9 moles percent (thermal denaturation) compared to 56.6 for the isolate of *S. marcescens*. These data indicate that the Gram-negative, pink-pigment-producing bacterium associated with crown rot of alfalfa is a strain of *S. marcescens*.

Shinde and Lukezic (20,21) noted that two groups of Gram-negative bacteria were commonly associated with a root and crown disease complex of alfalfa (*Medicago sativa* L.). One group consisted of green-fluorescent pseudomonads and the other fits the general description of the genus *Erwinia*. The erwinias appeared to belong to the group characterized by *Erwinia amylovora* as described by Dye (6) and were named *E. amylovora* var. *alfalfae*. The designation, *E. amylovora* var. *alfalfae*, was not included in the Approved Lists of Bacterial Names (24). This omission created a problem as to the proper designation of this pathogen. Recent reference to this group as "*Erwinia amylovora*-like" (8) suggests additional confusion as to its proper designation. Preliminary results suggested that these strains belonged in the genus *Serratia* rather than *Erwinia* because of the pigment production.

The investigations reported here were conducted to determine if the alfalfa erwinias should be placed in the genus *Serratia*.

## MATERIALS AND METHODS

The strains used in this study were: *Serratia marcescens* Bizio (strain SM-1) obtained from the Department of Bacteriology, University of California, Berkeley; *Erwinia rhapontici* (Millard) Burkholder (ER8D31) obtained from C. I. Kado, University of California, Davis; *Erwinia amylovora* (Burrill) Winslow et al, isolated from a blighted pear twig at University Park, PA; and the *Erwinia* strains isolated from diseased alfalfa plants. The exact sources of the alfalfa strains were: strain E1 (NCPPB 2641) and strain E2 (NCPPB 2642) were isolated from cultivar Buffalo plants grown near University Park, PA; strain E3 (ICPB EA217) was isolated from cultivar Cherokee plants grown near University Park; strain E4 was from plants of cultivar Niagara; and strains E5 and E6 (NCPPB 2643) were from plants of cultivar Buffalo grown near Allentown, PA. The original isolations were made in 1971 and 1972.

Nutrient yeast glucose calcium carbonate agar (NYDCA) (21) was used as a maintenance medium. Long-term storage was by lyophilization in skim milk. Misaghi and Grogan's (17) basic medium (supplemented with 0.05% nicotinic acid when appropriate) was used to determine carbohydrate utilization. The carbon-source compounds (0.2% w/v) were added aseptically after sterilization by membrane filtration. Pigment production was determined after 10 days of incubation on medium A of King et al (KA) (13). The methods to determine production of hydrogen sulfide, phenylalanine deaminase, indole, lysine, decarboxylase, ornithine decarboxylase; growth in the presence of potassium cyanide; and the methyl red and Voges-Proskauer (V-P) reactions were as described by Holding and Collee (12). However, for the methyl-red test the bacteria were grown in Dye's second methyl-red medium (6). Nitrate reduction was determined by using the method outlined by Bradbury (1). Gelatin liquefaction was determined after incubation at 25 C for 72 hr. Arginine dihydrolase production was tested by using Thornley's method (26). Citrate utilization and urease production were determined by the methods described by Skerman (23). The production of extracellular pectinase was determined with the different pectin gels as described by Hildebrand (10).

The serological methods used in the original description of the bacterial variety (21) were also used in this investigation.

The guanine-plus-cytosine content (in moles percent) of the DNA of the test strains was determined by the thermal denaturation method of Mandel and Marmur (16) with a Gilford Model 24005 spectrophotometer equipped with a thermal programmer. Normal probability paper (15) was used to establish the $\sigma$ value.

The ability of the organisms to induce a hypersensitive response in tobacco, *Nicotiana tabacum* L. 'Samsun,' Glurk and Turk, was tested by the technique outlined by Klement (14). To determine if the alfalfa strains and *S. marcescens* affected alfalfa seedling growth, surface-sterilized seeds of cultivar Buffalo, were inoculated with suspensions of the alfalfa strains and strain SM-1. The seeds were surface sterilized with 0.1% $HgCl_2$ in 50% ethyl alcohol (one volume of seeds in 10 volumes of liquid) for 20 min, and rinsed five times with sterile water. Three seeds were placed in capped, sterile, 2.5 × 15-cm test tubes containing about 30 g of sterile riverbank sand (autoclaved two separate times at 125 C for 30 min). The bacteria were grown on NYDCA for 24 hr, washed, and suspended in sterile water or Hoagland's #1 solution (11). The concentration of bacteria used, adjusted photometrically and verified by standard plate count procedures, was $3 \times 10^7$ colony-forming units per milliliter. Hoagland's solution was omitted to reduce host vigor to

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determine if nutrient-stressed plants were susceptible to the bacteria. Ten milliliters of the suspension was pipetted into each tube. Sterile Hoagland's solution or water was used as controls. Thirty-six plants were inoculated with each isolate. The tubes were left capped for 48 hr after inoculation, then were uncapped and exposed to a daily 16-hr photoperiod in a growth room at about 21 C. At the end of 24 days, the plants were washed and evaluated for damage by the organisms by counting the number of surviving plants and measuring plant height.

Characterization of the red pigment was completed from pigments produced in KA agar after 76 hr of incubation. Twenty plates each were inoculated with a highly concentrated water suspension of strains SM-1, E6, and *E. rhapontici*. Pigments were extracted by chopping the agar into pieces approximately 0.5 $cm^2$, which were in 200 ml of 95% ethanol. The mixture was shaken on a rotary shaker for 3 hr and centrifuged at 1,000 *g* for 10 min. Subsamples of the supernatant were spotted on filter paper sheets (Whatman 3 MM Chrom) and developed ascendingly with each of the following solvents: 95% ethanol; 100% ethanol; butanol-acetic acid-water (BAH) (3:1:1, v/v); phenol-$H_2O$ (3:1, w/v); and ethyl ether-petroleum ether (1:2, v/v).

The absorption spectrum of the pigment was determined after the extract was applied to the paper, developed with BAH, and eluted with BAH. Stability of the pigment in solutions of strong acid or alkali was determined after chromatographic separation with the pigments in BAH (27). No attempt was made to use a standard concentration of the pigments. One milliliter of a 1M solution of HCl or NaOH was added to 10 ml of the extract. The reactions were allowed to proceed for 1 hr before spectra were measured.

## RESULTS

The results of the biochemical reactions are summarized in Table 1. Serological tests with sonicated antigens resulted in an absence of bands in gel-diffusion plates among the alfalfa strains, *S. marcescens*, and *E. rhapontici*. There were at least four bands in the homologous interaction and in the interaction between different strains of the alfalfa isolates (Fig. 1).

The guanine plus cytosine (GC) content of the DNA from the alfalfa strains were 56.6 to 57.9 moles percent with a 2 $\sigma$ of 5.2 for E1 and 56.6 moles percent for SM-1 with a 2 $\sigma$ of 5.2. The ratio for *E. rhapontici* was 51.5 moles percent with a 2 $\sigma$ of 3.7. The reported values of the GC contents of the orgnisms of concern were, *S. marcescens* 53–59 moles percent, *E. rhapontici* 51.0–53.1 moles percent, and for *E. amylovora* 53.3–54.1 moles percent (2).

The hypersensitive reaction was not induced by the isolates of *S. marcescens* nor by the alfalfa isolates, but both *E. amylovora* and *E. rhapontici* induced the typical hypersensitive reaction in tobacco

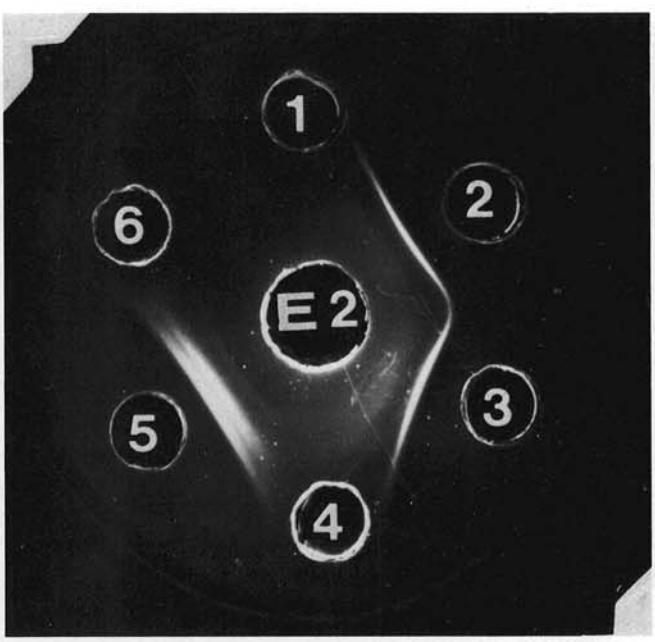


**Fig. 1.** Precipitin band pattern formed with antiserum against *Erwinia amylovora* var. *alfalfae* (E2) (center well) and homologous antigens and antigens from sonicated cells of *E. amylovora* var. *alfalfae* (E6), *Serratia marcescens*, and *E. rhapontici*. The saline control solution or antigens in the outer wells were: well 1, saline solution; well 2, E2; well 3, E6; well 4, *S. marcescens*; well 5, E2; and well 6, *E. rhapontici*.

TABLE 1. Comparison[v] of the biochemical reactions used to characterize *Serratia* and *Erwinia* spp.

| Substrate or test | *Serratia*[w] | *Serratia marcescens* (SM-1) | *Erwinia amylovora* var. *alfalfae* | *Erwinia amylovora* | *Erwinia rhapontici* |
|---|---|---|---|---|---|
| Glucose utilization | + | + | + | + | + |
| (gas) | d | − | − | − | − |
| Lactose | d | − | − | − | − |
| Sucrose | + | + | + | + | + |
| Maltose | + | + | + | + | − |
| Mannitol | + | + | + | + | + |
| Dulcitol | − | − | − | − | + |
| Rhamnose | − | − | − | − | + |
| Arabinose | − | +[x] | +[x] | − | − |
| Inositol | d | + | + | − | + |
| Xylose | d | + | + | + | + |
| Raffinose | − | − | − | − | + |
| Sorbitol | + | + | + | + | + |
| Adonitol | d | + | + | − | 0 |
| Trehalose | + | + | + | + | + |
| Cellobiose | + | + | + | + | + |
| Glycerol | + | + | + | + | + |
| Indole | − | − | − | − | − |
| Methyl Red | | | | | |
| 22 C | d | − | − | − | − |
| 37 C | d | − | − | − | − |
| Voges-Proskauer | | | | | |
| 22 C | + | + | + | + | + |
| 37 C | + | + | + | − | − |
| Citrate (Simmons) | + | + | + | + | − |
| Urease | d | + | + | − | − |
| $H_2S$ | − | − | − | − | − |
| Nitrite | + | + | + | − | + |
| Gelatin | + | + | + | + | − |
| Phenylalanine deaminase | − | − | − | − | − |
| Malonate | − | − | − | − | + |
| KCN broth | + | + | + | − | − |
| Lysine decarboxylase | + | + | + | − | − |
| Litmus milk | + | +[y] | +[y] | − | − |
| Arginine dihydrolase | − | − | − | − | − |
| Ornithine decarboxylase | + | + | + | − | − |
| Mucate | − | + | + | 0 | 0 |
| Ammonium salt | + | + | + | + | + |
| Glucose sugar | | | | | |
| Pigment | d | + | + | − | + |
| Motility | + | + | + | + | + |
| Pectate liquefaction | 0 | − | − | − | + |
| Induces hypersensitive responses | 0 | − | − | + | + |
| Growth | | | | | |
| 5 C | 0[z] | − | − | − | − |
| 40 C | + | + | + | − | − |

[v] Symbols: + = positive reaction in 1 or 2 days; − = no reaction or negative test; d = some strains positive, some negative; and 0 = test not completed.
[w] Data from Ewing et al (7).
[x] Delayed arabinose fermenters (positive after 5 days).
[y] Forms a clot.
[z] Data from Grimont et al (9).

TABLE 2. Influence of *Serratia marcescens* (SM-1), *Erwinia rhapontici*, and two strains of *E. amylovora* var. *alfalfae* and plant nutrition on percent survival and growth of alfalfa seedlings in a gnotobiotic system

| | Water only | | | Hoagland's solution[a] | | |
|---|---|---|---|---|---|---|
| Inoculated | Survival (%) | Top length (mm) | Root length (mm) | Survival (%) | Top length (mm) | Root length (mm) |
| Control | 61[b,c] | 24.7[d] | 64 | 64 | 57 | 65 |
| SM1 | 50 | 32.0 | 70 | 66 | 69 | 54.1 |
| *E. rhapontici* | 47 | 27.7 | 64 | 72 | 62 | 56 |
| E2 | 83 | 33.6 | 58 | 52 | 69 | 56 |
| E6 | 64 | 22.7 | 49 | 86 | 65 | 50 |

[a] Hoagland and Arnon (11).
[b] Each strain was inoculated onto 36 seeds (three seeds per capped tube).
[c] Means of measurements determined on surviving plants.
[d] Differences between the means were not statistically significant.

TABLE 3. Paper chromatography of ethanol-extracted bacterial pigments developed with different solvents

| | $R_f$[a] of pigments from: | | |
|---|---|---|---|
| Solvent system | *Serratia marcescens* (SM-1) | *Erwinia rhapontici* | *Erwinia amylovora* var. *alfalfae* (E6) |
| 95% Ethanol | 1 | 0.49[b] | 0.49[b] |
| 100% Ethanol | 1 | 0.50[b] | 0.46[b] |
| Butanol-acetic acid-$H_2O$ (3:1:1) | 0.91 | 0.46 | 0.50 |
| Phenol-$H_2O$ (3:1) | 0.96 | 0.85 | 0.88 |
| Ethyl-petroleum ether (1:2) | 0 | 0 | 0 |

[a] Developed on Whatman 3 MM Chrom paper.
[b] The spot formed a long tail.

TABLE 4. Comparison of spectral properties of prodigiosin from *Serratia marcescens* (SM-1), *Erwinia rhapontici*, and *Erwinia amylovora* var. *alfalfae* (E6)

| | Points for comparison | | | |
|---|---|---|---|---|
| | Minimum (nm) | Shoulder (nm) | Maximum (nm) | Return to base (nm) |
| E6 | | | | |
| Normal | 440 | 515 | 545 | 585 |
| Acid curve | | No peak | | |
| Alkaline curve | 435 | 515 | 550 | 600 |
| *E. rhapontici* | | | | |
| Normal | 430 | 525 | 565 | 615 |
| Acid curve | | No peak | | |
| Alkaline curve | 435 | 515 | 550 | 610 |
| SM-1 | | | | |
| Normal | 440 | ··· | 530 | 600 |
| Acid curve | 435 | ··· | 535 | 590 |
| Alkaline curve | 435 | ··· | 535 | 580 |

plants. Germination of alfalfa seeds, plant height, root length, and degree of root rot were not affected by the alfalfa isolates or *S. marcescens* isolates tested even when the plants were grown at a low level of nutrients (Table 2).

All the alfalfa strains except E4 produced a light-pink, diffusible pigment on medium A of King et al as previously reported (21). This pigment was lighter than the pink, diffusible pigment produced by *E. rhapontici* and very light compared to the dark-red pigment produced by *S. marcescens* (SM-1). The absorption spectra (Fig. 2), chromatographic data (Table 3), and reaction with strong acid (Table 4) showed that the pigments produced by the alfalfa strains and by *E. rhapontici* were identical in those characters examined. These pigments were different from the pigment produced by SM-1 because they had an absorption maximum at 545–565 nm and a shoulder at 515–525 nm, whereas the absorption maximum of SM-1 was 530 nm. The SM-1 pigment was resistant to strong acid, whereas the pigments from alfalfa isolates and *E. rhapontici* were destroyed.

## DISCUSSION

The physiological properties of the alfalfa strains fit the general description of *S. marcescens* (9), with a percentage of similarity (%S) of 95, calculated (17) according to the formula, $\%S = Ns/(Ns + Nd) \times 100$. The test strain of *S. marcescens* (SM-1) and the alfalfa strains were mucate-positive, while the results in the literature (7) report negative use of mucate. Arabinose was used by both SM-1 and the alfalfa strains but the positive results were evident only after 5 days.

The %S of the test strains of *E. amylovora* and the alfalfa strains was 71. The alfalfa strains differ because they use arabinose, inositol, adonitol, and mucate; reduce nitrate, clot litmus milk, and grow in a KCN broth. They were also V-P positive at 37 C, produce urease, lysine decarboxylase, ornithine decarboxylase, a pink diffusible pigment, and were able to grow at 40 C. With a %S of 58 it is very apparent that there were several differences between the alfalfa strains and the strains of *E. rhapontici*, yet they both produce similar if not identical pigments (Tables 2 and 4, and Fig. 2). The alfalfa strains differ from *E. rhapontici*; they utilized maltose, arabinose, and citrate, but could not utilize dulcitol, malonate, rhamnose, or raffinose. They grew at 40 C, grew in KCN broth, clotted litmus milk and liquified gelatin but not pectin. They were V-P positive at 37 C and produced urease, lysine decarboxylase, and ornithine decarboxylase. They also differed from *E. rhapontici* in being unable to induce a hypersensitive response in tobacco leaves.

The guanine-plus-cytosine contents of the DNA were in agreement with values reported for both *S. marcescens*, 53–59 moles percent, and *E. rhapontici*, 51.0–53.1 moles percent (2). These determinations show that the alfalfa strains are more closely related to the genus *Serratia* than *E. rhapontici* except for the similarity of the pigment produced by *E. rhapontici* and the alfalfa group. This factor cannot be resolved at the present time. However, members belonging to the genus *Serratia* share many common features with members belonging to the genus *Erwinia* (4,7,25).

The genus *Serratia* has been in a state of flux and only recently has been thoroughly delineated (9). For example, the eighth edition of Bergey's Manual of Determinative Bacteriology (2) reduced the initially recognized five species to one; however, a recent computer analysis of the genus (9) indicated that there are four phenons in *Serratia*, which correspond to the discrete species *S. marcescens*, *S. liquefaciens*, *S. plymuthica*, and *S. marinorubra*.

We have used the set of differential characteristics of Grimont et al (9) to classify the alfalfa strains. With this information as a basis, the alfalfa strains belong to Grimont's phenon A, which is typical of the species *S. marcescens*. P. A. D. Grimont (*personal communication*) has confirmed this classification and classified *E. amylovora* var. *alfalfae* strain E3 as *S. marcescens* biotype A 4a.

The inability to detect common antigens between the *S. marcescens* strains used and the alfalfa strains could be explained by observations of Davis and Woodward (5). They separated 16 strains of *Serratia* spp. into six distinct serological groups. However, they also observed that the pigmented strains tested were in two groups. Davis and Woodward warned that the sample tested

was small and that this observation may not hold for a larger sample. If Davis and Woodward were correct, then it is possible that the weakly pigmented alfalfa isolate belongs in a different serological group from the strongly pigmented *S. marcescens* that was tested.

The absence of symptoms on the inoculated alfalfa seedlings was unexpected because the organisms were reported to be virulent (21). However, loss of virulence by bacteria when cultured has been a serious enough problem to warrant several investigations (3). Another explanation may be the difference in experimental conditions. Shinde and Lukezic (22) reported interactions between these bacteria and other pathogens of alfalfa. Experimental conditions used in this research were modified to eliminate these types of interactions. It is possible that the presence of other microorganisms in previous tests influenced the presence of symptoms by providing avenues of entrance.

The occurrence of *S. marcescens* in the roots of alfalfa may have public health implications. There is accumulating evidence that strains of some of the so-called opportunistic pathogens such as *Pseudomonas aeruginosa* (18) and *Klebsiella pneumoniae* (19) occur in various environments including agricultural areas; many of the strains are indistinguishable from clinical strains.

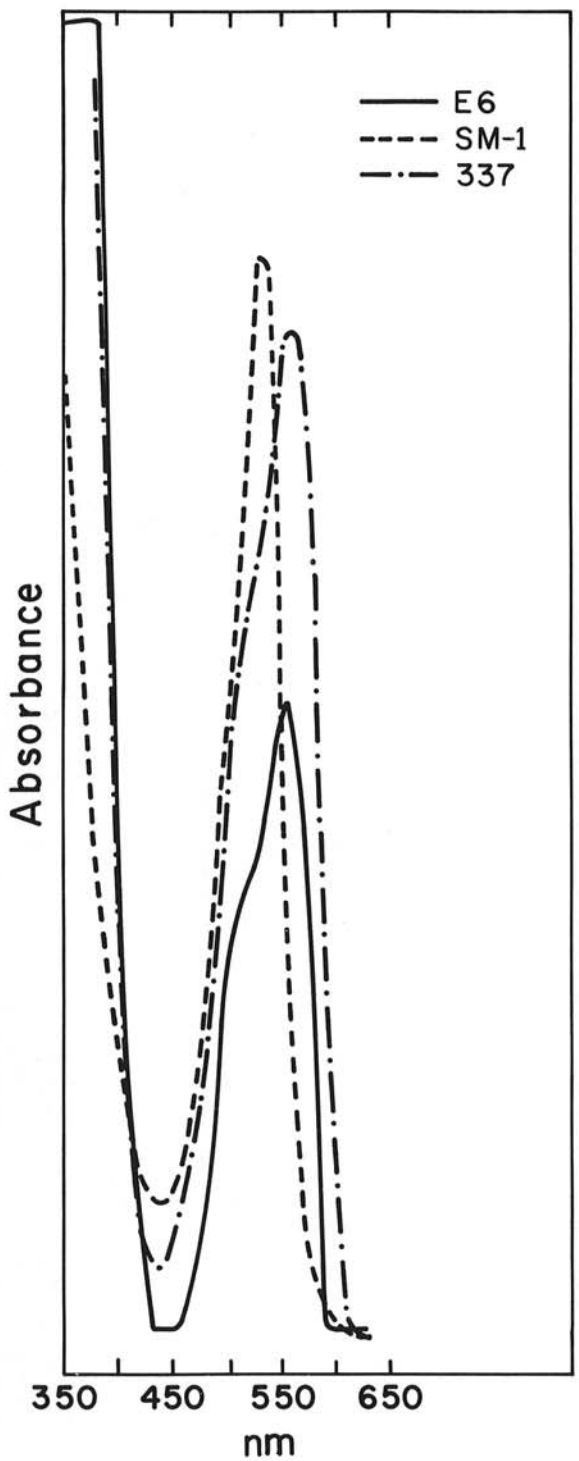


**Fig. 2.** Spectral properties of ethanol-extracted pigments. The pigments were chromatographed and eluted with butanol-acetic acid-water (3:1:1). The curves represent absorbance data for pigments from *Erwinia amylovora* var. *alfalfae* (E6), *Serratia marcescens* (SM-1), and *E. rhapontici* (337).

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