Pseudomonas corrugata, a Pathogen of Tomato, Isolated from Symptomless Alfalfa Roots

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ABSTRACT

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Pseudomonas corrugata, the causal agent of a serious disease of greenhouse-grown tomato plants in England, was isolated from healthy roots of greenhouse-grown alfalfa plants in the USA. Inoculations of bacterial strains from both alfalfa and tomato into tomato stems induced symptoms similar to those originally described for the disease. Strains from both sources caused localized necrosis when injected into alfalfa stems and roots, rotted onion scales and produced necrotic lesions on lettuce leaves, but potato tuber tissue was not rotted. Attempts to isolate strains of the organism from field-grown alfalfa plants failed. Therefore, its role in root and crown diseases of alfalfa was not determined. This is the first report on the occurrence of *P. corrugata* in the USA.

In 1974 I reported (10) the isolation of pathogenic bacteria resembling *Pseudomonas cepacia* from surface-sterilized cross sections of healthy alfalfa roots that were being used to study the interactions between strains of *Corynebacterium insidiosum*. The bacteria produced a dark blue pigment which was evident both in the colonies and in the root tissue beneath the colonies. That report listed some similarities with *P. cepacia*, such as production of yellow pigment, nonfluorescence, presence of poly-β-hydroxybutyrate granules, and showing an oxidase positive reaction. The alfalfa strains differed from *P. cepacia* in being unable to grow at 41 C and being able to break down gelatin rapidly.

A similar bacterial strain, isolated from a diseased tomato stem, was described by Scarlett, et al (15) who concluded that the new strain was different from the *P. cepacia* group. They established a new species, *Pseudomonas corrugata* Roberts and Scarlett, and designated isolate NCPPB 2445 as the type culture.

For this study an isolate of the type culture was obtained through the courtesy of Pauline Roberts.

This report is a summary of different biochemical, physiological, and pathogenicity test results which show the bacteria isolated from symptomless alfalfa roots to be identical with *P. corrugata.*

MATERIALS AND METHODS

Cultures.—Representative strains of the nonfluorescent, poly-β-hydroxybutyrate-accumulating pseudomonad group were compared in this study and their sources were: *P. caryophylli* strains PC 113 and PC 115, from the International Collection of Phytopathogenic Bacteria (ICPB), University of California, Davis; *P. cepacia* strains UCB 446 and UCB 459 from M. Schroth, Department of Plant Pathology, University of California, Berkeley; strain Pc C_2 and Pc 23 from ICPB; and the type strain, *P. corrugata* NCPPB 2445, from the National Collection of Plant Pathogenic Bacteria, Harpenden, England. Alfalfa strains PSU 297, 298, 299, 300, 309, 310, 384, and 386 were isolated from symptomless internal tissue of greenhouse-grown alfalfa roots.

Pseudomonas syringae, strains UCB 970 (from M. Schroth) and PSU 314 from Department of Plant Pathology, The Pennsylvania State University, were used as typical phytopathogenic fluorescent pseudomonads.

Pathogenicity tests.—For these tests, the bacteria that had been stored in sterile tap water were transferred to YDCA (glucose, 20 g; yeast extract, 10 g; $CaCO_3$, 20 g; and agar, 15 g; in 1 L of H_2O) and allowed to grow for 48 hr before use. The roots of 1-yr-old alfalfa plants (*Medicago sativa* L. 'Saranac') were freed of soil by washing. Three 5-mm diameter roots of each plant were inoculated by wounding with a teasing needle smeared with bacteria. Surface water from sterile medium was used as a control. Each isolate was inoculated into four separate plants which were transplanted into sterile sand and covered with a plastic bag for 24 hr. Five days later the roots were washed free of sand and evaluated for disease development.

The stems of greenhouse-grown alfalfa, tomato (*Lycopersicon esculentum* Mill.), and onion (*Allium cepa* L.) were inoculated by injection with a suspension of two of the alfalfa strains and NCPPB 2445. The inocula in tap water were adjusted to a constant level of 20% transmission at 420 nm ($\sim 10 \times 10^8$ cells per ml) before use. The plants were bagged for 24 hr and left on a greenhouse bench. Disease development was evaluated 5 days later by measuring the length of the necrotic lesions that had formed at the inoculation site.

The ability of the bacteria to rot potato tubers, onion bulbs, and lettuce leaves was determined. Squares of onion scales from a mature onion were placed, either with the adaxial or the abaxial side up, on 0.7% water agar and inoculated with a loopful of bacteria on the cut surface and upper side. Squares of lettuce leaves removed from the inner leaves of head lettuce were placed on moistened filter paper and inoculated on an uninjured site on the abaxial site. Sections removed from a sound, surface-sterilized potato tuber, were placed on 1.5% water agar. The tuber slices were not washed before inoculation because this process inhibits suberization (17). The potato slice was inoculated by placing a loopful of bacteria from a 24-hr culture grown on YDCA in the center of the slice. All of the plant material was kept in petri plates and incubated in the dark at 27 C. An extension of necrosis or soft rot at least 2.5 mm beyond the inoculation site was considered positive.

Biochemical and physiological tests.—Tests for the production of levan and 2-ketogluconate, the presence of arginine dihydrolase, nitrate reduction, and the liquefaction of gelatin were the same as those described by Misaghi and Grogan (13). The test for oxidase reaction was the same as Misaghi and Grogan, except that the bacteria were from 3-day-old cultures grown on the basic salts

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medium (plus 5.0 g glucose/L) formulated by Moustafa and Whittenbury (14). The oxidase reactions of strains grown on this medium were consistent whereas that of one strain was variable when it was grown on King's B Medium (6). The production of pectinases was determined in polypectate gels with three different pH ranges (4.9–5.1, 6.9–7.1, and 8.3–8.5) as described by Hildebrand (3) and tests for lecithinases and starch hydrolase were as described by Holding and Collee (4). The presence of lipase was determined with 1 g of polyoxyethylene sorbitan monolaurate (Tween-20) in a medium composed of 5 g peptone, 3 g yeast extract, and 2 g agar in 100 ml of tap water. The formation of a white precipitate surrounding the colony indicated presence of the enzyme. Tests for phenylalanine deamination were conducted as described by Skerman (16).

Nutritional tests were conducted with a basic medium that contained in 1 liter of glass distilled water: K_2HPO_4 (anhydrous), 3 g; NaH_2PO_4 (anhydrous), 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g: Bacto purified agar, 15 g (9). The pH was adjusted to 7 with 1N NaOH before autoclaving. A precipitate formed during autoclaving but this dissolved upon cooling. Other recommended media (13, 18) were tested in preliminary experiments but the above formulation produced comparable results and was simpler to make. Sugars and other substances likely to be decomposed by autoclavinng were sterilized by membrane filtration and added to the basal medium after autoclaving. The pH of the test compounds was adjusted with 1N NaOH or 1N HCl before filtration as needed. Heat-stable compounds were added to the basic medium and autoclaved for 20 min at 115 C. All test compounds were used at a concentration of 0.2% (w/v) with the exception of geraniol of which one drop was placed on the underside of the dish top.

After pouring, the plates were allowed to dry for 3 days at room temperature. The test organisms were grown on YDCA slants for 48 hr, then suspended in glass-distilled water to 40% transmission ($\sim 2 \times 10^7$ cells/ml). Seventeen strains were spotted on each plate with a 1-mm diameter platinum loop. Each test included several plates of YDCA and plates of the basic salts medium with and without glucose as carbon source controls. Plates were incubated at 27 C in the dark and were evaluated for growth 2, 5, and 7 days after inoculation. The compounds were considered to have been utilized when the colonies were larger than colonies grown on the control medium without a carbon source.

Pigment production and colony formation.—Pigment production by the test strains on alfalfa (cultivar Saranac) roots was detected by inoculating cross sections cut from 5 mm or larger diameter roots of 6-mo-old plants. Before they were sectioned, the roots were surface-sterilized with 70% ethanol for 5 min, rinsed, soaked in 0.5% sodium hypochlorite for 2 min, and rinsed again. The sections were placed in 0.7% water agar in petri plates and a suspension of the bacteria in tap water was placed on the cut surface and incubated at 27 C for 72 hr. By this time, a characteristic blue pigment was easily detectable. Because *P. lemonnieri,* a soil bacterium, also produces a blue pigment which is affected by medium composition and has some cultural similarity to the alfalfa strain and *P. corrugata,* a medium described by Starr et al (19) was used to determine if pigment production by the test strains was affected similarly. The medium, containing 5% peptone and 2% agar in tap water, was used to determine the effect of glucose (0.5, 1.0, 2.0, 3, and 5 g per 100 ml) on pigment production. Pigment production was evaluated 36-48 hr after inoculation. Pigment characterization by the method described by Starr et al (19) was attempted. King's A Medium and King's B Medium (6) and a 4% glycerin-beef extract-agar for *Pseudomonas pseudomallei* (2) also were used to detect pigment production.

Temperature relationships.—Bacteria from 48-hr-old cultures on King's B Medium were streaked on slants of the same medium and incubated at 34, 36, 37, 39, 40, 41, and 42 C. If growth was not evident after 72 hr, the material from the slant surface was streaked on fresh slants and incubated at 27 C for 48 hr and reexamined for growth.

Hypersensitivity of tobacco.—Leaves of *Nicotiana glutinosa* L. Glurk and Turk were infiltrated with a bacterial suspension containing approximately 8×10^7 cells/ml, returned to the greenhouse bench, and evaluated 24 hr later (7).

Determination of base composition of DNA.—The moles % of guanine plus cytosine (% GC) in the DNA were determined by the purification procedure of Marmur (12) and the ultraviolet absorbance-temperature profile method of Mandel and Marmur (11). The thermal melting values were calculated as described by Knittel et al (8). DNA from *Serratia marcescens* (strain SM-1 obtained from M. Schroth) was used as a control.

Cytological observations.—The pattern of flagellation was determined by electron microscopic observation of bacteria from 18- to 24-hr-old nutrient dextrose broth cultures (Difco nutrient broth 8 g/L; dextrose 10 g/L) and negatively stained with 1.0% phosphotungstic acid at pH 7.0. The accumulation of poly-β-hydroxybutyric acid was detected by staining smears with Sudan

TABLE 1. Comparison of biochemical and physiological traits of *Pseudomonas syringae*, *P. caryophylli*, *P. cepacia*, *P. corrugata*, and the alfalfa strains of *P. corrugata*

Comparative tests	Nomenspecies				
	P. syringae	*P. caryophylli*	*P. cepacia*	*P. corrugata*	Alfalfa strains
	1[a]	2	4	1	8
Potato soft rot	−	−	−	−	−
Onion rot	−	−	+	+	+
Lettuce necrosis	+	+	+	+	+
Tobacco hypersensitivity (7)[b]	+	+	+	+	+
Pigment on alfalfa roots	−	−	−	+	+
Oxidases (13)	−	+	+	+	+
Proteases	−	+	+	+	+
Lecithinases (4)	−	+	+	−	−
Lipases (16)	−	+	+	+	−
Arginine dihydrolase (13)	−	+	−	−	−
DL-phenylalanine deaminase (16)	−	−	+	−	−
Starch hydrolase (4)	−	−	−	−	−
Pectinase (3)	−	−	+	−	−
2-keto-gluconate (13)	−	+	−	−	−
Levan (13)	−	+	−	−	−
Max. temperatures (C)	36	>40	>40	38	38
Poly-β-hydroxybutyrate	−	−	+	+	+
Nitrate reduction (13)	−	−	−	+	+
Growth at pH 4.5	−	−	−	−	−

[a]Number of isolates tested.
[b]Literature reference to method.

TABLE 2. Utilization of carbohydrates, alcohols, polyalcohols, and glycols by *Pseudomonas syringae*, *P. caryophylli*, *P. cepacia*, *P. corrugata*, and the alfalfa strains of *P. corrugata*

Compounds tested[a]	Nomenspecies				
	P. syringae 2[b]	*P. caryophylli* 2	*P. cepacia* 4	*P. corrugata* 1	Alfalfa strains 8
Carbohydrates:					
Glucose	+	+	+	+	+
Rhamnose	−	+	+	−	−
Sucrose	+	+	+	+	+
Maltose	−	−	−	+	d(2)[c]
Trehalose	−	+	+	+	+
D-Fucose	−	ND[d]	ND	−	−
L-Fucose	−	−	−	−	−
D-Galactose	+	+	+	+	+
D-Arabinose	−	−	−	−	−
L-Arabinose	+	+	+	+	+
Cellobiose	−	−	−	−	−
β-Lactose	−	−	−	−	−
Alcohols:					
Ethanol	d (1)	−	−	−	−
Geraniol	−	−	−	−	−
Polyalcohols and glycols:					
Arabitol	−	+	+	−	−
Erythritol	+	−	−	−	−
Meso-inositol	+	−	+	+	+
Mannitol	+	−	+	+	+
Sorbitol	+	−	+	−	−

[a] All test compounds were added at a concentration of 0.2% (w/v) with the exception of geraniol which was placed dropwise on the underside of the dish top, to a basic medium consisting of K_2HPO_4 (anhydrous), 3 g; NaH_2PO_4 (anhydrous), 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$ or 3 g; Bacto purified agar 15 g in 1 L of glass distilled water. The pH was adjusted to 7 before autoclaving.
[b] Number of isolates tested.
[c] Different cultures gave different reactions (number of cultures that were positive appear in parentheses).
[d] Determination not made.

TABLE 3. Utilization of amino acids and other nitrogenous compounds by *Pseudomonas syringae*, *P. caryophylli*, *P. cepacia*, *P. corrugata*, and the alfalfa strains of *P. corrugata*

Compound tested[a]	Nomenspecies				
	P. syringae 2[b]	*P. caryophylli* 2	*P. cepacia* 4	*P. corrugata* 1	Alfalfa strains 8
Amines:					
Putrescine	ND[c]	ND	ND	+	+
Spermine	ND	ND	ND	+	d (6)[d]
Aliphalic amino acids:					
Alanine	ND	ND	−	ND	+
δ-Amino-N-valeric acid	ND	−	+	+	+
Arginine	+	−	ND	ND	+
Asparagine	ND	−	ND	+	d (6)
Betaine	−	+	+	+	d (2)
L-Histidine	+	+	+	+	+
Homoserine	−	−	ND	−	−
L-Lysine	ND	−	+	−	d (2)
Serine	−	−	+	−	−
D-L-Threonine	−	−	−	−	−
L-Threonine	−	−	−	−	−
Aromatic amino acids:					
Phenylalanine	ND	ND	ND	ND	+
Miscellaneous nitrogenous compounds					
Acetamide	−	d (1)	+	−	−
Nicotinate	−	−	−	−	−
Uracil	−	−	−	−	d (1)

[a] All test compounds were added at a concentration of 0.2% (w/v) to a basic medium consisting of K_2HPO_4 (anhydrous), 3 g; NaH_2PO_4 (anhydrous), 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$ or 3 g; Bacto purified agar 15 g in 1 L of glass distilled water. The pH was adjusted to 7 before autoclaving.
[b] Number of isolates tested.
[c] Determinations not made (ND).
[d] Different cultures gave different reactions (number of cultures that were positive appear in parentheses).

Black (1). The bacteria were grown on the basic mineral medium with $(NH_4)_2SO_4$ at a low concentration (0.2 g/L) and DL-β-hydroxybutyrate (5 g/L) as a carbon source; otherwise, the technique was the same as described by Stanier et al (18).

RESULTS

Pathogenicity tests.—The strain of *P. corrugata* and the alfalfa strain, caused small necrotic lesions in wound-inoculated alfalfa roots which extended approximately 5 mm beyond the inoculation site. The strain of *P. syringae* did not cause necrosis. Strains of *P. cepacia* varied; two isolates caused necrosis and one did not. In alfalfa stems, all of the strains caused necrotic lesions. The alfalfa strains and *P. corrugata* caused necrotic lesions on tomato stems. Necrosis in the pith usually extended 1.5 cm from the inoculation site; however, wilting had not occurred at the time of evaluation.

The strains of *P. syringae* and *P. caryophylli* did not cause rot of potato and onion (Table 1). As expected, *P. cepacia* strains caused rot in potato tuber slices and in the cut surface of onion bulb scales but did not do so in the latter if placed on the unwounded abaxial or adaxial surfaces. All of the alfalfa strains and *P. corrugata* rotted onion pieces when inoculated onto the cut surfaces. Three of the alfalfa strains rotted onion pieces when placed on the abaxial side and one isolate caused rot when placed on the adaxial side. All of the strains caused necrosis of uninjured lettuce tissue.

Pigment production.—The blue pigment produced by the bacteria on the glucose-peptone-agar diffused into the agar, but diffusion was restricted and the color was not apparent until the blue colonies were washed off. The limited ability to diffuse into the agar suggested restricted water solubility. The pigment was not soluble in methanolic hydrochloric acid or chloroform.

Production of the blue pigment was influenced by the glucose concentration of the medium; 1% glucose was optimal whereas concentrations of 0.5% and 2% or above reduced the amount of pigment produced. The blue pigment faded after 48 hr. On peptone-glucose medium, at the 1% glucose level, a diffusible dull orange pigment was observed under a long wavelength (320–380 nm) ultraviolet light. As the blue pigment faded, the orange pigment changed to a yellow color.

On different batches of the yeast extract-glucose-carbonate agar the strains formed convex rugose colonies. This character was not stable, upon repeated culturing the colonies became smooth convex and were yellow-orange. No diffusible pigment was produced on King's A or B Medium by the alfalfa strains and *P. corrugata*. On King's B Medium the colonies were light yellow and convex.

Hypersensitivity response.—All of the strains elicited the hypersensitive response on both cultivars of tobacco.

Cytological observations.—All the alfalfa strains had three to six polar flagella and were rods measuring 1.8–2.4 × 0.6–1.2 μm. Apparently the bacteria accumulated poly-β-hydroxybutyrate, but in some strains only in a few cells.

Percentage of similarity.—The percentage of similarity (% S) was calculated with the formula of Misaghi and Grogan (13). They considered a test to be positive when 90% of the strains of a nomenspecies were positive. The % S of *P. corrugata* and the alfalfa strains was 94.1. The % S of the alfalfa strains and *P. cepacia* was 48.8; for *P. caryophylli* it was 48.8, and for *P. syringae,* 80.6.

DISCUSSION

The bacterial strains from alfalfa were closely similar if not identical with *P. corrugata*, which causes tomato pith necrosis in England (15). The results of this investigation support the establishment of a new species for this group of strains. As reported by Scarlett et al (15), the major differences that separate these organisms from *P. cepacia* and its relatives in the nonfluorescent group of plant pathogenic pseudomonads are: a lower maximum temperature for growth, pectin liquefication, formation of mucoid colonies on certain media, and the differential utilization of carbon sources. In addition, a % GC ratio of 57–59 for the alfalfa strains compared to 65 for *P. caryophylli* and 66–68 for *P. cepacia* (Table 1) also supported this concept. Unfortunately the strain of *P. corrugata* was not received in time to determine the % GC for comparison and the information is not available from the literature.

The production of the blue pigment and the influence of medium composition on its production suggested that the alfalfa strains may be similar to *P. lemonnieri* which was isolated from soil. Fortunately, an extensive characterization of strains belonging to this species was completed by Stanier et al (18) and Hugo and Turner (5). Based on their information, a % S of 74 between the alfalfa strains and *P. lemonnieri* was derived. The major differences were that cultures of *P. lemonnieri*: produced a fluorescent water soluble pigment on King's B Medium and on potato tissue, produced levan, did not accumulate poly-β-hydroxybutyrate, and had a maximum temperature for growth of 30 C. The solubility of the blue pigment produced by *P. lemonnieri* was different from that of the pigment produced by the alfalfa isolates.

The % GC ratio determined for the alfalfa strains was very close

TABLE 4. Utilization of organic acids by *Pseudomonas syringae*, *P. caryophylli*, *P. cepacia*, *P. corrugata*, and the alfalfa strains of *P. corrugata*

Compound tested[a]	Nomenspecies				
	P. syringae 2[b]	*P. caryophylli* 2	*P. cepacia* 4	*P. corrugata* 1	Alfalfa strains 8
Glycolate	−	−	+	+	−
Lactate	−	+	+	+	+
Adipate	−	−	+	−	−
Glutarate	−	−	−	+	+
Hydroxysuccinate	−	−	ND[c]	+	+
Malonate	+	ND	+	+	+
D-Tartrate	−	−	−	−	−
L-Tartrate	−	−	+	−	d (2)[d]
M-Tartrate	−	+	+	−	−
Citraconate	−	−	−	−	−
Mesaconate	−	−	−	−	−
D-Gluconiate	+	+	ND	+	+
O-Hydroxybenzoate	−	−	−	−	−
P-Hydroxybenzoate	−	−	−	−	−
Poly-β-hydroxybutyrate	+	+	+	+	+

[a]All test compounds were added at a concentration of 0.2% (w/v) to a basic medium consisting of K_2HPO_4 (anhydrous), 3 g; NaH_2PO_4 (anhydrous), 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$ or 3 g; Bacto purified agar 15 g in 1 L of glass distilled water. The pH was adjusted to 7 before autoclaving.
[b]Number of isolates tested.
[c]Determinations not made (ND).
[d]Different cultures gave different reactions (number of cultures that were positive appear in parentheses).

to the ratio of 59–60 determined for *P. syringae* (Table 1). However, as with *P. lemonnieri,* there were enough idfferences between *P. syringae* and the alfalfa strains (% S of 80.6) to consider them definitely different. Interestingly, the % S for *P. syringae* and *P. corrugata* was higher than that for *P. cepacia* which suggested a closer relationship to *P. syringae.*

The results of pathogenicity tests on young tomato and alfalfa plants demonstrated that strains from both hosts caused necrosis on either plant. Scarlett et al (15) reported that *P. corrugata* did not rot onion slices; however, in my test, that bacterium was capable of rotting onion sections if inoculated onto a cut surface.

The role, if any, of the alfalfa strains in the root and crown rot complex is not known at this time. Several attempts to isolate these organisms from healthy and diseased field-grown alfalfa plants have failed. Symptoms similar to those described by Scarlett et al (15) have not been observed in greenhouse-grown tomato plants at several locations in Pennsylvania.

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