Ecology and Physiology of Fluorescent Pectolytic Pseudomonads

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ABSTRACT

A selective medium was used to isolate fluorescent pectolytic pseudomonads from potato tubers and potato rhizospheres, leafy plant tissue, soil, sewage, and garbage. One-hundred forty isolates were subjected to 21 biochemical tests and evaluated for ability to macerate potato tissue. No single biochemical test or group of tests could be used to differentiate isolates that macerate from those which do not. Potato-macerating isolates among the fluorescent pseudomonads represented a continuum of phenotypes from *Pseudomonas fluorescens* to *Pseudomonas putida*. Fluorescent pseudomonads were commonly isolated from soft rots of potato tuber, but rarely were found in the soil obtained from potato fields at planting time. Examination of 18 foundation seed piece sources showed that 15 contained fluorescent pseudomonads able to macerate potato tissue.

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Pectolytic bacteria are commonly implicated in soft rot diseases of plants. If isolates are gram-negative motile rods and produce a yellow-green fluorescent pigment, they are designated as fluorescent pseudomonads. There is no reliable way, other than ability to macerate the host tissue, to differentiate these fluorescent isolates, from the ubiquitous nonmacerating fluorescent saprophytes. One exception is *Pseudomonas viridiflava* which is oxidase-negative (8, 14). Fluorescent pectolytic bacteria from plant soft rots are currently grouped as either *Pseudomonas fluorescens,* sometimes as biotype II of that species (1, 2, 6, 8), or as *P. marginalis* (1, 8). Pseudomonads associated with pink-eye disease of potato are generally classified as *P. fluorescens* (2). Those isolated from leafy plants are commonly classed as *P. marginalis* (8).

We wanted to determine (i) the degree of variability among the fluorescent pectolytic pseudomonads from plants, and (ii) if the results of biochemical tests could be correlated with the ability to macerate potato tuber tissue. Such biochemical tests would aid diagnosis of plant diseases and facilitate the selective breeding of disease-resistant plants.

Additionally, soil populations of fluorescent pseudomonads fluctuate widely, depending on the state of decomposition of soil organic matter (10). Information about their ecology could lead to more effective control of the diseases caused by these plant pathogens.

MATERIALS AND METHODS.—*Sources of seed pieces and soil.*—Seed pieces were obtained from foundation sources at planting time. Soil samples from the top 15 cm were obtained from potato fields on the planting date.

Analysis of potato tissue.—About 10% of each seed piece was removed, weighed, and then ground in a sterile mortar with a volume of 0.1M potassium phosphate solution (pH 6.8) equal to the weight of potato tissue. The solids were allowed to settle and 0.1 ml of the supernatant fluid was spread on freshly poured solid FPA medium (12). This medium, which is selective for pseudomonads, contains the antibiotics cycloheximide, penicillin, and novobiocin (13) in addition to pectin (12). Additionally, dilution series of the original supernatant fluid were made in sterile distilled water and also plated. Plates were incubated for 48 hours at 30 C and fluorescent colonies were observed with ultraviolet light (366 nm) and marked. The plates were then flooded with 1% hexadecyltrimethylammonium bromide (Fisher Chemical Co., Fairlawn, N.J.) as previously described (5). Colonies surrounded by a clear halo in an opaque medium are pectolytic. Colonies which were both fluorescent and pectolytic were counted.

Isolations from natural materials (114 isolates) were also made on the FPA medium. Colonies were picked from plates after observation of halos around the colonies (less than 5 minutes) and retested as pure cultures on the same medium.

Analysis of soil.—A 10-g sample of soil with 100 ml of distilled water in a 240-ml bottle was shaken for 15 minutes. After the solids had settled, 0.1 ml samples were plated on FPA medium. Fluorescence and pectolytic activity were determined as previously described.

Biochemical tests.—To detect enzyme production, maceration, and biochemical activity by isolates, the bacteria were first grown for 24 hours at 30 C in shake culture in a mineral salts solution (19) that contained 1.5% glycerol. An aliquot of this culture fluid was then suspended in sterile distilled water to provide a just-visible turbid solution (approximately 10^6 cells/ml). One drop of this suspension was placed directly on the hardened media and plates incubated at 30 C. Each isolate was tested at least twice.

The medium of Hankin et al. (5) was used to detect pectate transeliminase production. Testing of isolates in liquid culture and subsequent spectrophotometric analysis confirmed the ability of this medium to detect production of pectate transeliminase (20). After incubation for 48 hours, the plates were flooded with a 1% solution of hexadecyltrimethylammonium bromide to detect pectolytic activity. To detect extracellular protease production, the bacteria were inoculated on nutrient agar (Difco, Detroit, Mich.) to which a sterile gelatin solution

was added to provide a concentration of 0.4% in the medium (16). After 48 hours of incubation, plates were flooded with a saturated solution of ammonium sulfate to differentiate zones of proteolysis around colonies.

A medium described by Sierra (15) was used to detect lipase production. Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Chem., Industries, Wilmington, Del.) was the lipid source. To detect cellulase (C_x) production the medium of Hankin et al. (4) was used with pectin omitted and carboxymethylcellulose (Cellulose gum, type 120 high, Hercules) added to provide a 1% final concentration. Details for the use of these media are described in Hankin et al. (3, 4).

Maceration was tested on potato disks (10 mm in diameter and 1-2 mm thick) cut from the inner parenchyma of Katahdin potato tubers that had been washed, dipped in alcohol, and flamed. The disks were washed several times in distilled water, placed in a solution of 0.52% sodium hypochlorite, a 10% solution of household bleach (Clorox), for 3 minutes as adapted from Melouk and Horner (9). The solution was decanted, and the disks were washed three times with sterile distilled water.

Three disks were then placed in a petri dish with 1 ml of sterile distilled water and inoculated with one drop of a test suspension that contained 10^6 bacterial cells/ml. Only isolates that macerated potato tissue disks within 48 hours were considered positive.

TABLE 1. Source, number, and potato tissue macerating ability of isolates of fluorescent pseudomonads

Source of isolate	Macerating isolates (no.)	Nonmacerating isolates (no.)
Potato tuber	72[a]	20
Potato rhizosphere	8	0
Other leafy plants	17[b]	2
Soil	16	2
Sewage sludge	2	0
Garbage	0	1

[a]Includes nine macerating isolates from Maine.
[b]Includes eight macerating isolates from England.

TABLE 2. Biochemical tests which were variable among 140 cultures of fluorescent pseudomonads and percentage of positive reactors divided into macerating and nonmacerating isolates of potato tissue

Test	Macerating isolates (% +)	Nonmacerating isolates (% +)	Difference (%)
Fluorescence	98	88	10
Protease production	93	61	32[a]
Lipase production	82	76	6
Sensitivity to norvaline	80	69	11
Denitrification (growth)	79	61	18
Levan production	78	46	32[a]
Pectate transeliminase production	78	26	52[a]
Inositol utilization	75	50	25
Sensitivity to ethionine	67	25	41[a]
Ethanol utilization	67	26	41[a]
Sensitivity to methionine sulfoximine	52	88	−36[a]

[a]Tests most useful for differentiating macerating from nonmacerating isolates.

Denitrification and the production of levan from sucrose was tested by the method of Stanier et al. (17). Glucose fermentation was detected by the method of Hugh and Leifson (7), and Thornley's 2A medium (18) was used to detect arginine dihydrolase. Fluorescence was observed under ultraviolet light (366 nm) after 48 hours of growth on Agar F (Difco).

Sensitivity to amino acid analogs was tested on a medium that contained mineral salts (19), 1.5% glycerol, and 1.1% Ionagar 2 S (Wilson Diagnostics, Chicago, Ill.). Analogs used were: 4 aza-DL-leucine dihydrochloride (ICN, Cleveland, Ohio); DL-methionine-DL-sulfoximine (3-amino-3-carboxypropyl-methyl sulfoximine) (Sigma, St. Louis, Mo.); 5-methyl-DL-tryptophan (Sigma), DL-norvaline (Sigma); L-ethionine (Sigma); D-cycloserine (Sigma) and DL-p-fluorophenylaline. Solutions were prepared by adding 0.1 ml ethanol per mmole of analog. After 10 minutes at room temperature, these solutions received 10 ml of sterile 0.1 M potassium phosphate buffer, pH 6.8, per mmole of analog. A 0.1-ml aliquot of this solution was placed on a filter paper disk (12.7 mm diameter, Schleicher and Schuell No. 740E). Thus, each disk contained 10 μmoles of the analog. Each analog was prepared as described above except for methionine sulfoximine. For this analog 20 ml of the phosphate buffer was used, therefore only 5 μmoles of methionine sulfoximine were placed on each disk.

The disks were then placed on the surface of plates of the test medium which had been surface-inoculated with single isolates. After they were incubated for 48 hours at 30 C, the plates were observed for zones of inhibition.

Utilization of ethanol was tested with a medium that contained mineral salts (19), 1.1% Ionagar, and 6% ethanol. The ethanol was added to the previously sterilized and cooled agar medium just before the plates were poured. Utilization of DL-sorbitol (Sigma) and m-inositol (Sigma) was tested on the mineral salts-agar medium to which a solution of 2% (w/v) of filter-sterilized sorbitol or inositol was added to provide a final concentration of 0.2% in the medium. Growth for all these tests was compared to that on the same agar medium lacking the carbon source.

For detecting the production of cytochrome oxidase, day-old cells from Agar F plates were smeared onto filter paper previously wet with an oxidase reagent (17) and observed for the characteristic blue color reaction within 30 seconds.

RESULTS.—The source and macerating ability of the 140 isolates is shown in Table 1. Potato disks were macerated by 115 of these isolates. Most macerating isolates were from Connecticut, but eight were from England and nine from Maine. Based on initial assays, all were classified only as fluorescent pseudomonads with pectinase activity. To test if the isolates comprised a compact group, and to determine if any physiological or biochemical tests correlated with ability to macerate plant tissue, 22 biochemical tests were applied to each isolate.

Of the 22 biochemical tests applied to the 140 isolates, nine tests gave the same response for all isolates. These were production of arginine dihydrolase; inability to ferment glucose; inability to produce cellulase (C_x) and amylase; sensitivity to four amino acid analogs,

cycloserine, methyltryptophan, azaleucine, and fluorophenylalanine; and a positive response to the oxidase test (one exception). Only five isolates did not fluoresce. These five isolates were originally fluorescent, but apparently lost this characteristic on subculture as has been previously described (17).

All isolates were classified on the basis of their ability to macerate potato tissue. Table 2 shows the 12 variable tests and the numbers indicate percentage of isolates positive for designated tests. For example, 93% of all macerating isolates were able to produce a protease.

Those isolates that responded identically to certain tests were grouped. In this way 72 different groups were formed. The largest group comprised 34 identical isolates. This group (represented by No. 134 in Table 3) was positive for all tests and sensitive to all analogs except methionine sulfoximine. These isolates were all obtained from potatoes or potato rhizospheres and might constitute a "potato" group. Of the 72 groups, only 64% were able to macerate potato tissue whereas of the 140 isolates tested, 89% were able to do so. This discrepancy between data on groups and isolates is due primarily to the large single group of 34 identical potato isolates.

An attempt was made to differentiate the biochemical responses of individual isolates according to source of isolation. The 92 isolates from potato tuber and eight from potato rhizosphere included 27 different groups of macerators and 20 groups of nonmacerators. Of 21 isolates from other plant tissue, 16 were able to macerate potato tissue. Of these, all except one were able to excrete pectate transeliminase, and all except one produced a protease.

Sixteen of the 18 isolates from soil were able to macerate potato tissue and only 14 of these were also able to excrete pectate transeliminase. All except one of the macerating isolates from soil produced a protease, and all except one gave a positive test for denitrification. The isolates from a secondary sewage treatment facility (3) were able to macerate potato tissue and excreted pectate transeliminase. They differed from each other in seven of the 22 biochemical tests.

To determine the ecological sources of fluorescent pseudomonads both potato seed pieces and soil were examined. Seed pieces from Connecticut, Maine, Minnesota, and New York carried fluorescent pectolytic pseudomonads. Nearly half of the pieces examined (31 of 72) contained these bacteria. We assayed four replicate samples of soil from each of seven potato fields obtained at planting time and in only one sample from only one field were a few fluorescent pectolytic pseudomonads found. The limit of detection for the assay used is 2×10^2 organisms per gram soil.

DISCUSSION.—The most comprehensive attempt to define groups within the genus *Pseudomonas* was made by Stanier et al. (17) and revised recently by Doudoroff and Palleroni (1). They determined five groups of fluorescent pseudomonads with many biotypes within these groups, and observed a wide diversity among their strains. Our data also show considerable diversity. To illustrate this, 10 macerating isolates from potato were selected (Table 3) to represent almost the complete continuum of phenotype from *P. fluorescens* (No. 135) to a P. putida-like organism (No. 111). Note that No. 135 is positive for all tests, and that No. 111 is negative for all tests except sensitivity to methionine sulfoximine. The enigma is that these 10 isolates (and those they represent) are all able to macerate potato tissue. Apparently no one variable character tested is essential to maceration and it probably would not be enough for a host plant to block the activity of a protease, pectinase, or lipase in order to be resistant to attack by the bacterium. There is the possibility that an organism could produce one of these enzymes only when grown in host tissue. This may explain why some of our isolates macerate tissue, but do not produce pectate transeliminase on test media. Some evidence for this is shown by Hankin et al. (5) who found that extracts of potato when added to solid media enhance production of this enzyme.

Overall, the 115 strains able to macerate potato tissue fit the pattern for two of the usual diagnostic tests for *P. fluorescens;* i.e., 98% were fluorescent and 93% were proteolytic. However, only 79% were denitrifiers, 78% produced levan, and 67% utilized ethanol. These latter three tests when positive are diagnostic tests for *P. fluorescens* biotype II, the group in which *P. marginalis* strains are placed (1). Although a majority of our isolates fall within the *P. fluorescens* group, some strains are similar to *P. putida;* i.e., those strains that do not denitrify, produce levan, or protease, and many strains are intermediate in phenotype.

Although the variability among isolates is considerable (Table 3), we have reason to believe that even if more

TABLE 3. Pseudomonad isolates[a] from potato selected to show variability in response to 11 biochemical tests

Biochemical tests	Isolate no.									
	111	160	138	141	20	14	24	102	134	135
Ethanol utilization	−	−	−	−	−	+	+	+	+	+
Levan production	−	−	−	+	−	−	+	+	+	+
Sorbitol utilization	−	−	−	−	−	+	+	+	+	+
Sensitivity to ethionine	−	−	−	−	+	+	−	+	+	+
Inositol utilization	−	−	−	−	+	+	+	+	+	+
Denitrification (growth)	−	+	−	+	−	−	+	+	+	+
Pectate transeliminase production	−	−	+	−	+	+	+	−	+	+
Sensitivity to norvaline	−	−	+	+	+	+	+	+	+	+
Lipase production	−	+	+	+	+	+	−	+	+	+
Protease production	−	+	+	+	+	−	+	+	+	+
Sensitivity to methionine sulfoximine	+	+	+	+	+	+	−	−	−	+

[a]All 10 isolates were capable of macerating potato tissue.

isolates were studied, all possible combinations of characteristics would not be found. For example, one might expect to find "either/or" situations. That is, if an organism lacks an alanine permease, it must synthesize alanine; similarly, if it lacks the ability to synthesize alanine, it must have an alanine permease. Hence, one biochemical-genetic deletion excludes and prohibits another. Such an "either/or" situation apparently exists with the 140 strains studied in their sensitivity to ethionine and methionine sulfoximine. Strains are either sensitive to both, or resistant to either, but we did not find resistance to both. For example, of 115 strains that were able to macerate potato tissue (Table 1), 21 were resistant to ethionine, and 57 were resistant to methionine sulfoximine. None was resistant to both analogs and 31 were sensitive to both.

Stanier et al. (17) found that fluorescent pseudomonads isolated from soil were usually *P. putida;* i.e., they did not produce proteolytic enzymes and were not denitrifiers. Our soil isolates which are proteolytic may represent a residual population (10) carried into the soil on plant material or in water, and are not soil inhabitants. Some of our isolates are from soils where pink-eye diseases were found in previous growing seasons. Folsom and Friedman (2) observed that pink-eye disease of potato caused by *P. fluorescens* often is abundant where soil-moisture content was high, and absent where the soil was very dry.

Our quantitative data on contaminated seed pieces differ from those of Sampson and Hayward (11) in Australia. They sampled only internal tissue, and we sampled a corner of the seed piece. Our concern was whether the seed piece was contaminated, and not the exact location of the source of infection. Although our data suggest that soft rot bacteria are transmitted via seed pieces, other unexplored modes of transmission, such as insects or alternate hosts, may also be important.

The question remains as to why apparently healthy potatoes that contain pectolytic pseudomonads do not rot. The biochemical mechanisms within the tissue that prevent or inhibit growth of pectolytic pseudomonads, and thus prevent rotting of the tissue, have yet to be fully explained.

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