Comparison of Plant-Pathogenic Pseudomonads by Disc-Gel Electrophoresis

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Oregon Agricultural Experiment Station Technical Paper No. 2985. Supported in part by the Oregon State Prune Commission.

Accepted for publication 18 March 1971.

ABSTRACT

Thirty-eight isolates representing *Pseudomonas syringae, P. morsprunorum, P. phaseolicola, P. lachrymans, P. glycinea, P. fluorescens,* and *P. aeruginosa* were compared using disc-gel electrophoresis and physiological and pathogenicity tests. Electrophoretic data confirmed the close relationship among the oxidase-negative plant pathogens, as well as their distinction from the saprophytic species. Protein band patterns for *P. glycinea, P. phasiolicola,* and *P. morsprunorum* were quite similar, and could be distinguished from the other species by the occurrence of a densely staining band above the two frontal bands. Patterns of *P. syringae* and *P. lachrymans* were similar in many respects, and were distinguishable from the others. *Pseudomonas fluorescens* and *P. aeruginosa* were distinct from any of the other isolates tested. The data support the placing of the oxidase-negative plant pathogens in a single taxospecies. However, distinctions within this group can be made at the subspecies level by some physiological tests and gel electrophoresis. Phytopathology 61:984-986.

Additional key words: Prunus, cherry, prune, pear.

New techniques and the accumulation of knowledge have led to numerous changes in the taxonomy of phytopathogenic bacteria. Current studies tend to group into fewer species-related organisms that might have formerly merited separate specific rank. Nutritional and biochemical tests (13, 16) have shown the close relationship that exists among the oxidase-negative phytopathogenic pseudomonads. *Pseudomonas syringae* and *P. morsprunorum* form a heterogeneous complex within this group, and have been studied extensively (2). Phage typing (7), serology (1, 14, 17), and pathogenicity have all been used for distinguishing between the two species and for dividing them into recognizable groups at the intraspecific level.

In recent years, gel electrophoresis of soluble proteins has been increasingly used as a taxonomic tool in the study of microorganisms, with particular application to plant pathogens (6, 8, 9, 10). The purpose of this study was to make an electrophoretic comparison of isolates of *P. syringae* from a variety of hosts and locales as well as to compare them with other pathogenic and saprophytic fluorescent pseudomonads in order to provide new information concerning their relationships.

MATERIALS AND METHODS.—*Cultures.*—Cultures were obtained from other laboratories or were isolated from diseased trees in the Willamette Valley of Oregon (Table 1). Each isolate was purified by streaking on King's medium B (12). Single colonies were selected from plates with homogeneous colonies and used to prepare stock cultures which were stored according to the method of DeVay & Schnathorst (5). Pathogenicity of *P. syringae* and *P. morsprunorum* isolates was confirmed by inoculating green fruits of sweet cherry and Italian prune. Other pathogenic species were not checked for pathogenicity on their respective hosts, but all were oxidase-negative, and caused a hypersensitive reaction in tobacco.

Electrophoresis.—Cultures for electrophoretic studies were grown for 3 days at 25 C in 1-liter flasks containing 400 ml of nutrient glucose broth (3 g beef extract, 10 g peptone, 10 g glucose/liter). Cultures were checked for purity at harvest by streaking a sample from each flask on a plate of medium B agar. Cells were harvested by centrifuging for 10 min at 5,800 *g*, washing in 50 volumes of 0.05 M Tris [tris(hydroxymethyl)amino methane]-HCl at pH 7.2 and centrifuging again. Preliminary experiments comparing Tris, HEPES (*N*-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid), and phosphate buffers over a range of pH were conducted to determine the conditions to use.

Soluble proteins were extracted by suspending cells in equal volumes of buffer and sonicating with an MSE Ultrasonic Disintegrator for 3.5 min. To prevent heating during sonication, the suspension was surrounded by dry ice and sonicated for 20-sec periods, alternating with 5- to 8-sec pauses for cooling. The broken cell suspension was centrifuged at 4 C for 60 min at 100, 300 *g* in a Spinco Model L ultracentrifuge. The supernatant was divided into 0.5-ml aliquots and frozen at −20 C until needed.

Acrylamide gels were prepared according to the method of Davis (4) in 0.5-cm (internal diameter) × 8.0-cm tubes. Each tube contained ca. 500 µg protein as determined by the method of Lowry et al. (15). Bovine serum was used as the standard.

Electrophoresis was conducted at 2.0 ma/tube for 5 min, then at 4.6 ma/tube until the bromphenol blue front had moved about 4 cm through the running gel. Gels were stained for 25 min with Coomassie Brilliant Blue R 250 (3), destained for 2 days in 10% trichloroacetic acid, then transferred to 7% acetic acid for permanent storage. Densitometer tracings were made with a Schoeffel Model SD-3000 spectrodensitometer set at a wavelength of 650 nm.

Comparisons were made between duplicate gels using the same extract, using portions that were frozen for different lengths of time, and using different extraction

TABLE 1.

Host	Isolates[a]	Obtained from
	Pseudomonas syringae	
Cherry	940-R[b], 919-S[b]	California
	Ps-144[b]	Hungary
	43-1, 43-2, SC-1, SC-2, SC-4	Oregon
	GW-4, GW-8, GW-9, GW-13	Oregon
Prune	GS-3S[b], 7f-S1[b], 857-S2[b]	California
	GG-1, GG-2, Fr. prune	Oregon
Pear	P-2, P-3	Oregon
	B-310[b], E-3[b]	England
	S-3[b]	California
Peach	B-3[d], B-3AS[b]	California
Almond	912-S2[b]	California
Lilac	LM-1	Oregon
Bean	HP-C[b]	Wisconsin
?	Syr D[c]	Missouri
	Pseudomonas morsprunorum	
Plum	m.p.[c]	England
	B-299[b]	England
	P. phaseolicola	
Bean	9-B[e]	New York
	21-B[e]	New York
	SV-2[e]	Oregon
Cucumber	*P. lachrymans*	Oregon
Soybean	*P. glycinea*[c]	Missouri
	P. fluorescens	Oregon
	P. aeruginosa	Oregon

[a] Cultures not designated were isolated by the authors or obtained from others at Oregon State University, Corvallis.
[b] Obtained from Jack Otta, University of California, Davis.
[c] Obtained from R. N. Goodman, University of Missouri, Columbia.
[d] Obtained from J. E. DeVay, University of California, Davis.
[e] Obtained from E. K. Vaughan, Oregon State University, Corvallis.

from the same isolate. In addition, band positions and intensities of 1:1 protein mixtures of paired isolates were compared with those of each individual isolate (11). In this way, homologous bands (i.e., bands with the same migration velocity) were established, even though there were small uncontrollable differences in the actual migration velocities in some of the gels. After two or more widely separated pairs of bands per pair of gels had been established, the densitometer tracings were adjusted photographically to show them at equivalent points. A band-by-band comparison could then be made.

RESULTS.—Soluble protein extracts separated into ca. 25 bands after electrophoresis. Small, uncontrollable differences in migration velocities occurred in some tubes, but duplicates of the same extract, run at the same time, produced identical patterns. In the solutions frozen for several months, some bands did not stain as intensely as in fresher material. In addition, some bands lying close together did not separate as distinctly in older material as they did in fresher samples. Both fresh and older extracts could be readily identified as having come from the same isolate, however.

A comparison between samples of the same isolate cultured under different conditions (48 hr at 30 C vs. 72 hr at 25 C) also revealed similar patterns. The variation was no greater than that shown by samples frozen for different lengths of time.

In band-by-band comparisons, all of the named isolates of *P. syringae* as well as those assumed to be *P. syringae* on the basis of pathogenicity, host, and a few physiological tests, had very similar patterns (Fig. 1). There were some differences among isolates, especially in relative intensity of some bands, but these differences could not be correlated with either the host, geographical location, or, in those cases where it was known, the serotype.

The band patterns of the three isolates of *P. phaseolicola* were identical. The patterns of two isolates of *P. morsprunorum* were also identical to each other and distinct from *P. syringae* (Fig. 2). The most notable distinction was the presence in *P. morsprunorum* of a broad, densely staining band next to the second band from the anodal end of the gel. The same band was also present in *P. glycinea* and *P. phasiolicola* but absent in *P. lachrymans*.

Comparison of protein patterns from *P. syringae*, *P. morsprunorum*, *P. lachrymans*, *P. glycinea*, and *P. phaseolicola*, plus 1:1 mixtures of these paired in all possible combinations, revealed that the patterns for these species were similar. Most of the bands in each species could be matched with bands in each of the others. Differences were great enough, however, to en-

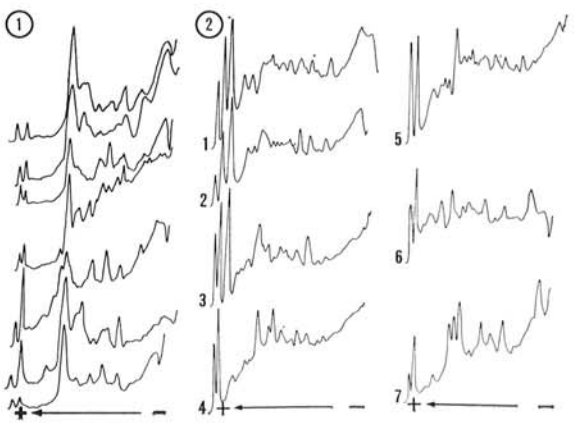

Fig. 1-2. Densitometer tracings of gels from *Pseudomonas* isolates. + and — indicate anode and cathode ends, and the arrow indicates the direction of protein movement through the gel. **1)** Tracings of *P. syringae* isolates from different hosts. Top to bottom: Fr. prune, prune; GS-13; cherry; 912-S2, almond; HP-C, bean; LM-1, lilac; E-3, pear; B-3AS, peach. Note that some differences in band patterns exist, but major differences are in relative densities of some bands. **2)** Tracings of patterns from different species of *Pseudomonas*. (1) *P. glycinea;* (2) *P. phaseolicola;* (3) *P. morsprunorum;* (4) *P. syringae;* (5) *P. lachrymans;* (6) *P. fluorescens;* (7) *P. aeruginosa*. Note the prominant band, third from the left in the first three species, that is absent in the others.

able a clear distinction to be made among these species. Patterns for *P. fluorescens* and *P. aeruginosa* were clearly different from the phytopathogenic species as well as from one another (Fig. 2).

DISCUSSION.—The value of electrophoretic data depends upon the procedure and methods used in interpreting the results (18). For example, uncontrollable variation in migration rates between tubes presents a problem when attempting to determine which bands constitute homologues. This is especially critical when comparing gels from separate runs. E_F values for a number of gels were calculated, but the results were completely unsatisfactory. Based on E_F values, there was greater variation among isolates of *P. syringae* than between *P. syringae* and the other phytopathogenic nomenspecies. Consequently, evaluation of results by the method of Johnson et al. (11) was found to give more satisfactory results.

Our results are in general agreement with others in indicating that a clear separation at the nomenspecies level can be made by means of electrophoresis. Therefore, this method should be of value as a relatively rapid and simple means of identification of species of *Pseudomonas*. Among the isolates of *P. syringae,* however, no differences could be detected that could be correlated with pathotype, serotype, or original host.

Electrophoretic data are significant in the light of other data concerning relationships among the species tested. Some maintain that *P. syringae* and *P. morsprunorum* constitute a single species. This view is supported by serological data in which Lovrekovich et al. (14) found that certain isolates of the two species were of the same serotype. Burki (1), on the other hand, found that a distinction could be made between the two species on the basis of flagellar antigens, but not O antigens. In addition, Burki (1) and Garrett et al. (7) showed from pathogenicity and biochemical and physiological tests that separation of the two is justified. Our data show that each of the five species tested, including *P. morsprunorum,* differed from all others by the presence of 5-6 nonhomologous bands between each pair of species. Based upon our electrophoretic evidence and that of others (1, 7, 14), *P. morsprunorum* appears to be an acceptable nomenspecies.

Electrophoretic data confirm the close relationships reported for the oxidase-negative plant pathogens, as well as their distinction from the saprophytic species (16). There were about twice as many nonhomologous bands between *P. fluorescens* or *P. aeruginosa* and the pathogenic species as there were between the different pathogens.

Based on the close biochemical relationships demonstrated by other workers and confirmed here by electrophoretic patterns, we support the placement of *P. syringae, P. morsprunorum, P. lachrymans, P. phaseolicola, P. glycinea,* with the other oxidase-negative plant pathogens in a single taxospecies. However, the distinction between nomenspecies within this group, which is very useful in plant pathology, can be made by means of gel electrophoresis.

LITERATURE CITED

1. BURKI, T. 1968. Untersuchungen an Obstbaum-pathogenen Pseudomonas-Arten der Schweiz. Schweizerische landwirtschaftliche Forschung VII:215-265.
2. CAMERON, H. R. 1962. Diseases of deciduous fruit trees incited by Pseudomonas syringae van Hall. Oregon Agr. Exp. Sta. Bull. 66. 62 p.
3. CHRAMBACH, A., R. A. REISFELD, B. WYCKOFF, & J. ZACCARI. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. 20:150-154.
4. DAVIS, B. J. 1964. Disc electrophoresis II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
5. DEVAY, J. E., & W. C. SCHNATHORST. 1963. Single-cell isolation and preservation of bacterial cultures. Nature 199:775-777.
6. EL-SHARKAWY, T. A., & D. HUISINGH. 1968. Identification of Xanthomonas species by acrylamide gel disc electrophoresis of soluble bacterial proteins. Phytopathology 58:1049 (Abstr.).
7. GARRETT, CONSTANCE M. E., C. G. PANAGOPOULOS, & J. E. CROSSE. 1966. Comparison of plant pathogenic pseudomonads from fruit trees. J. Appl. Bacteriol. 29:342-355.
8. GILL, H. S., & M. N. KHARE. 1968. Disc electrophoresis of sonic extracts of five phytogenic bacteria. Phytopathology 58:1051 (Abstr.).
9. HOITINK, H. A. J., K. J. HAGEDORN, & ELIZABETH MCCOY. 1968. Survival, transmission, and taxonomy of Pseudomonas syringae van Hall, the causal organism of bacterial brown spot of bean (Phaseolus vulgaris L.). Can. J. Microbiol. 14:437-441.
10. HUISINGH, D., & R. D. DURBIN. 1967. Physical and physiological methods for differentiating among Agrobacterium rhizogenes, A. tumefaciens, and A. radiobacter. Phytopathology 57:922-923.
11. JOHNSON, B. L., D. BARNHART, & O. HALL. 1967. Analysis of genome and species relationships in the polyploid wheats by protein electrophoresis. Amer. J. Bot. 54:1089-1098.
12. KING, ELIZABETH O., MARTHA K. WARD, & D. E. RANEY. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
13. LELLIOTT, R. A., EVE BILLING, & A. C. HAYWARD. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
14. LOVREKOVICH, L., Z. KLEMENT, & W. J. DOWSON. 1963. Serological investigation of Pseudomonas syringae and Pseudomonas morsprunorum strains. Phytopathol. Zeitschrift 47:19-24.
15. LOWRY, O. H., NIRA J. ROSEBROUGH, A. L. FARR, & ROSE J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
16. MISAGHI, I., & R. G. GROGAN. 1969. Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology 59:1436-1450.
17. OTTA, J. D., & H. ENGLISH. 1969. Pathological and serological delimitation of Pseudomonas syringae. Phytopathology 59:1043 (Abstr.).
18. SHIPTON, W. A., & G. FLEISCHMANN. 1969. Taxonomic significance of protein patterns of rust species and formae speciales obtained by disc electrophoresis. Can. J. Bot. 47:1351-1358.