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Genetic Diversity of *Pseudomonas solanacearum*: Detection of Restriction Fragment Length Polymorphisms with DNA Probes That Specify Virulence and the Hypersensitive Response

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Restriction fragment length polymorphism (RFLP) analysis involving nine DNA probes, seven of which encode information required for virulence and the hypersensitive response (HR), was used to study the relationships among 62 strains of *Pseudomonas solanacearum*, representing three races and five biovars. When Southern blots of *Eco*RI- or *Eco*RI and *Bam*HI-digested genomic DNA were probed with the nine unique *Eco*RI cloned fragments from strains K60 or K2R, 28 distinct RFLP patterns were identified. Similarity coefficients revealed two major divisions: division I contains all members of race 1 biovars 3, 4, and 5; division II contains all members of race 1 biovar 1 and races 2 and 3. Similarity coefficients within divisions I and II are 78% ± 9% and 62% ± 19%, respectively. In contrast, the similarity coefficient between the two divisions is only 13.5%. Division II is composed of five distinct subdivisions corresponding to race 1 biovar 1, race 3, and three subdivisions of race 2. Some subdivisions correspond to strains with distinct host ranges or geographical origins.

Additional keyword: bacterial wilt.

Pseudomonas solanacearum E. F. Smith, causal agent of bacterial wilt, is responsible for severe losses to many important crops in tropical, subtropical, and warm, temperate regions of the world. The species comprises a large and complex taxonomic group of strains, a fact that is reflected in the two systems of classification that have been employed in attempts to organize the species. Buddenhagen *et al.* (1962) divided the species into three races based on host range and colony morphology. Race 1 strains are pathogenic on a wide range of hosts such as peanut, ginger, olive, tobacco, potato, tomato, and diploid bananas. Race 2 strains are limited to musaceous hosts, including *Heliconia* spp. and triploid banana (*Musa* AAA), and race 3 primarily affects potato. More recently, two new races have been proposed for strains with host range limited to mulberry (He *et al.* 1983) and ginger (Pegg and Moffett 1971). It is clear, however, that host range is often an ambiguous and unreliable taxonomic character. Nonetheless, the race system of classification has proven valuable in differentiating strains with similar colony morphologies.

In a second classification scheme, Hayward (1964) separated the species into four biovars based on differences in the oxidation of three disaccharides (maltose, lactose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol). However, with the exception of biovar 2 (which corresponds well with race 3), there is no correlation between these particular physiological characteristics and host range (Hayward 1964; Palleroni and Doudoroff 1971; Harris 1972; Buddenhagen 1985). Race 1, which has a wide host range, includes some members of biovar 1 and all members of biovars 3 and 4, whereas race 2 includes the remaining members of biovar 1.

Other methods of classification have been used in attempts to describe the diversity of strains within *P. solanacearum*, but none has been successful. For example, Palleroni and Doudoroff (1971) compared DNA homology among 26 strains representing the four biovars and found no correlation with host range. Likewise, the use of monoclonal or polyclonal antisera (E. Barlow, unpublished data; Morton *et al.* 1966) has been ineffective for determining either race or biovar.

The apparent complexity and worldwide distribution of *P. solanacearum* has prompted questions as to whether it evolved separately in different geographical locations (Buddenhagen 1985). The many initial reports of bacterial wilt following the introduction of exotic crop plants in virgin areas indicate that *P. solanacearum* probably is endemic on the native flora in many parts of the world (Kelman 1953).

The situation with respect to strains of race 1 is complex because their host range and original geographic origin are uncertain. Race 1 strains are present worldwide on a broad range of hosts. A primary criterion for classifying strains as race 1 has been the host of origin, but host range determinations indicate considerable pathogenic diversity even among strains isolated from the same host.

In Central and South America, race 2 of *P. solanacearum* is endemic on *Heliconia* species in virgin jungle areas (Buddenhagen 1960; Sequeira and Averre 1961). Following the introduction of susceptible bananas and related *Musa* species, Moko disease became a serious problem. Several strains within race 2 have been described, but the relationships among these strains remain obscure (French and Sequeira 1970).

Race 3 is the most homogeneous group within *P. solanacearum* in terms of host range and physiological parameters and is nearly synonymous with biovar 2. Race 3 strains are limited primarily to potato and are thought to have evolved on native tuber-bearing *Solanum* species in the Andean region of South America. Although it is likely that

race 3 occurs in many potato-growing areas of the world because it was distributed in infected tubers, there is speculation that it may have originated outside of South America (Buddenhagen 1985; Seneviratne 1969).

Despite continued efforts to unravel the relationships among members of *P. solanacearum*, no substantial progress has been made since the implementation of the race and biovar systems of classification. Questions regarding the origin and evolution of *P. solanacearum* remain unanswered. Modern molecular biology techniques, however, provide the means to examine the relatedness of bacterial strains on a more exact basis (Gabriel *et al.* 1988). The objective of this study was to determine whether a classification of 62 strains of *P. solanacearum* could be developed based on analysis of restriction fragment length polymorphisms (RFLPs). RFLPs result from the addition or deletion of restriction endonuclease sites in the DNA of the bacterium, or from genomic rearrangements that alter the proximity of these sites. This variation, which is characteristic of each strain, can be detected by Southern blot analysis when cloned DNA fragments are used as probes. Results indicated that RFLP analysis can be used to predict the race and biovar of individual strains of *P. solanacearum* and that it may provide a basis for an analysis of the evolution and geographic distribution of this species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were from the collection maintained at the Department of Plant Pathology, University of Wisconsin, Madison. The original source of all strains is listed in Table 1. Bacterial strains were maintained, long term, as suspensions in sterile water and were retrieved by plating on tetrazolium chloride (TZC) medium (Kelman 1954). Colonies exhibiting the characteristic fluidal morphology of *P. solanacearum* were restreaked and used to prepare lyophilized stocks in a medium of 7% peptone and 7% sucrose. These lyophilized stocks were used as a backup source for each strain. Ordinarily, strains were grown at 28° C on TZC medium or in casamino acids, peptone, and glucose (CPG) broth (Hendrick and Sequeira 1984) on a rotary shaker at 170 rpm.

DNA probes. The nine DNA probes used in this study (Table 2) were obtained from other studies on the genetics of virulence of *P. solanacearum* and induction of the hypersensitive reaction (HR). All nine probes carry the transposon Tn*5*, which does not hybridize to DNA of *P. solanacearum*. Probes PT161 and PK700 carry transposons that affect tryptophan and extracellular polysaccharide (EPS) biosynthesis, respectively (Morales *et al.* 1985; Xu *et al.* 1988). The remaining seven probes encode functions required for both virulence and the HR, previously designated as *hrp* genes (Lindgren *et al.* 1986).

DNA manipulations. To obtain genomic DNA, a cell suspension (1.5 ml) from a 36-hr CPG culture was pelleted by centrifugation and resuspended in 500 ml of Tris HCl (50 mM, pH 7.5) buffer containing EDTA (10 mM) and NaCl (10 mM). Bacteria were lysed by adding Sarkosyl and proteinase K to 1% and 100 μg/ml, respectively, and incubating the mixture at 37° C until clear. The mixture was made 2 M with respect to ammonium acetate and then extracted twice with phenol and once with chloroform. DNA was precipitated with an equal volume of isopropanol and resuspended in 50 μl of TE buffer (10 mM Tris HCl, pH 8; and 1 mM EDTA). This preparation was used directly for digestion with the restriction enzyme *Eco*RI, or with a mixture of *Eco*RI and *Bam*HI. The procedures for agarose gel electrophoresis and Southern blotting were as described by Maniatis *et al.* (1982). Probe DNA was labeled with ^{32}P-dATP by nick translation according to manufacturer's instructions in a kit purchased from Bethesda Research Laboratories (Gaithersburg, MD). Hybridizations and subsequent washes were conducted by the method of Amasino (1986). Restriction fragments that hybridized to the probe DNA were detected by autoradiography on X-ray film.

Similarity coefficients. Similarity coefficients for all pairwise combinations of RFLP groups were calculated by means of the formula:

$$(2Nxy)(100) / (Nx + Ny)$$

in which Nx and Ny represent the number of unique restriction fragments describing organism x or y, respectively, and Nxy is the number of fragments common to both x and y (Nei and Li 1979). The three strains that lack homology with various probes (RFLP groups 7, 14, and 20; Table 3) were omitted from similarity coefficient calculations.

Pathogenicity and HR tests. For pathogenicity tests, triploid banana (*Musa* AAA) plants were grown for 4 wk at 28° C under fluorescent lighting in a growth chamber. Inoculum consisted of a bacterial suspension (5×10^8 cfu/ml) prepared from a 24- to 36-hr CPG broth culture. Plants were inoculated by puncturing the pseudostem, near the soil line, with a micropipette containing 50 μl of bacterial suspension. Control plants were inoculated with either strain UW 70 (race 2) or strain UW 25 (race 1). When inoculated with the virulent UW 70, wilt symptoms appeared within 2 wk. Plants inoculated with UW 25, which is not pathogenic to bananas, remained symptomless.

The ability of strains of *P. solanacearum* to induce the HR was assessed as described by Lozano and Sequeira (1970). Tobacco plants (*Nicotiana tabacum* 'Bottom Special') were grown at 28° C under fluorescent lighting in a growth chamber. Bacterial suspensions, prepared as described above, were infiltrated into the intercostal tissue of fully expanded tobacco leaves. Typical HR symptoms, consisting of confluent collapse and necrosis of the infiltrated tissue, appeared within 24 hr postinoculation.

Biovar analysis. Strains were classified into biovars based on their ability to oxidize three disaccharides (lactose, maltose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) (Hayward 1964). With this system, biovar 1 failed to oxidize either group of carbohydrates, biovar 2 oxidized the disaccharides but not the hexose alcohols, and biovar 4 oxidized the hexose alcohols but not the disaccharides. Biovar 3 oxidized both the hexose alcohols and the disaccharides; biovar 5 had a similar pattern, except that it failed to oxidize dulcitol and sorbitol (He *et al.* 1983).

RESULTS

Of the 38 race 1 strains tested, 13 belonged to biovar 1,

nine to biovar 3, 14 to biovar 4, and two to biovar 5 (Table 1). All 17 race 2 isolates belonged to biovar 1; the seven race 3 strains were classified as biovar 2 (Table 1). Similar associations of race and biovar have been reported previously, but exceptions apparently exist (Tabei and Quimio 1978; Hayward 1964).

A summary of the RFLP patterns observed in Southern blots of *Eco*RI- or *Eco*RI and *Bam*HI-digested DNA is presented in Table 3. Together, the nine probes used defined 28 unique RFLP groups, each group representing strains

Table 1. List of 62 strains of *P. solanacearum* used for RFLP analysis

UW number	Race	Biovar	Location	Host	RFLP group	Alternate strain numbers	Source
153	1	1	Australia	Potato	1	0158, S246	Hayward
30	1	1	Trinidad	Tomato	1	K136	Dudman
25	1	1	United States	Tomato	1	K60	Kelman
134	1	1	Kenya	Potato	1	S221, K197	n.a.[a]
26	1	1	United States	Tomato	2	K74	Kelman
258	1	1	Costa Rica	Potato	3		Sequeira
273	1	1	Costa Rica	Potato	3	G-16	Gonzalez
278	1	1	Mexico	Tobacco	3		Fucikovsky
277	1	1	Mexico	Banana	3		Fucikovsky
256	1	1	Costa Rica	Potato	4	G-7	Gonzalez
275	1	1	Costa Rica	*Melampodium perfoliatum*	5		Sequeira
154	1	1	Colombia	Tobacco	6	S247	Granada
90	1	1	Brazil	Tobacco	7	ENA521	Robbs
255	1	3	Costa Rica	Pepper	8	G-1	Gonzalez
130	1	3	Peru	Tomato	9	S225	Sequeira
143	1	3	Australia	Tomato	10	002a, S236	Hayward
152	1	3	Australia	Potato	10	0131a, S245	Hayward
380	1	3	China	Olive	11	OPS2	He
374	1	4	China	Peanut	11	PPS6	He
197	1	4	Philippines	Ginger	11	TA-1	Zehr
356	1	4	China	Eggplant	11	EPS1	He
369	1	4	China	Peanut	11	PPS14	He
198	1	3	Philippines	Potato	11	LB-6	Zehr
147	1	3	Australia	Tobacco	12	0170,S240	Hayward
8	1	3	Costa Rica	*Eupatorium odoratum*	13	S213, K201	Sequeira
119	1	3	Costa Rica	Potato	14	S213	Gonzalez
74	1	4	Sri Lanka	Potao	15	CMI #B2861	n.a.[a]
360	1	4	China	Mulberry	16	MPS5	He
375	1	4	China	Peanut	16	PPS9	He
376	1	4	China	Peanut	16	PPS1	He
364	1	4	China	Tomato	16	TMPS1	He
27	1	4	United States	Tobacco	17	K105	Kelman
378	1	4	China	Olive	18	OPS1	He
361	1	5	China	Mulberry	19	MPS4	He
373	1	5	China	Mulberry	20	MPS2	He
141	1	4	Australia	Ginger	21	007a, S234	Hayward
151	1	4	Australia	Ginger	22	092, S244	Hayward
359	1	4	China	Ginger	23	ZPS1	He
138	2	1	Costa Rica	Plantain	24	H7551-2, S231	Berg
167	2	1	Costa Rica	Banana	24	K160	Sequeira
9	2	1	Costa Rica	*Heliconia*	24	S147	Sequeira
28[b]	2	1	Cyprus	Potato	24	K118	Dawson
139	2	1	Costa Rica	Plantain	24	H7551-1, S232	Berg
135	2	1	Honduras	Banana	24	H5513-4, S228	Berg
136	2	1	Costa Rica	*Heliconia*	24	H189, S229	Berg
161	2	1	Peru	Plantain	25	S254	Sequeira
160	2	1	Peru	Plantain	25	S253	Sequeira
175	2	1	Colombia	Plantain	25	B-H1504, K250	Buddenhagen
162	2	1	Peru	Plantain	25	S255	Sequeira
70	2	1	Colombia	Plantain	25	S210	Thurston
127	2	1	Peru	Plantain	25	S222	Sequeira
128	2	1	Peru	Plantain	25	S223	Sequeira
129	2	1	Peru	Plantain	25	S224	Sequeira
73	3	2	Sri Lanka	Potato	26	CMI #B2768	n.a.[a]
150	3	2	Australia	Potato	26	0137a, S243	Hayward
145	3	2	Australia	Potato	26	018a, S238	Hayward
23	3	2	Israel	Potato	26	K56	Volcani
81	3	2	Colombia	Potato	27	S207	Thurston
80	3	2	Colombia	Potato	27	S206	Thurston
19	3	2	Colombia	Potato	27	S205	Thurston
181	2	1	Venezuela	Plantain	28	158, K261	Buddenhagen
20	2	1	Venezuela	Banana	28	S215,K260	Buddenhagen

[a] n.a., data not available.
[b] UW28 is a misidentified strain that was previously classified as race 1 and reclassified as race 2 based on pathogenicity tests and RFLP patterns.

with identical RFLP patterns. Each of the three races and four biovars are composed of multiple RFLP groups. No RFLP group includes members from more than one race or biovar, with the exception of RFLP group 11, which includes strains of biovars 3 and 4 (Table 1). Consequently, the race to which any isolate belongs can be determined, and biovars 1, 2, 5, and the combined 3/4 can be distinguished from each other.

Similarity coefficients, calculated for all pairwise combinations of the 28 RFLP groups, indicated that *P. solanacearum* can be separated into two distinct divisions that have an average similarity coefficient (S) of 13.5% (Table 4). For example, Southern blot analysis of *Eco*RI-digested DNA probed with pT34 (Fig. 1) allows separation of all strains into two primary groups. The only two exceptions are RFLP groups 19 and 20, which give a unique pattern with this probe and are coincidentally the only representatives of biovar 5 used in this analysis. In hybridization tests with *Eco*RI-digested DNA, only one probe, pK700, fails to distinguish between these two divisions. With *Eco*RI and *Bam*HI-digested DNA, however, the same probe allows separation of the species into the two divisions (Table 3). Division I (S = 78 ± 9%) includes all members of race 1 biovars 3 and 4 and the two race 1 biovar 5 mulberry strains (RFLP groups 19 and 20) (Table 1). Division II (S = 62 ± 19%) includes the race 1 biovar 1 strains and all members of races 2 and 3 (Table 1).

Sixteen RFLP groups can be distinguished among the 25 strains that make up division I. In most cases, distinction between RFLP groups in division I can be made on the basis of patterns observed with three probes: 34, 600, and 140 (Table 3); the remaining six probes generally fail to discriminate between these strains. RFLP groups 11 and 16 represent 40% of all strains within division I and have a similarity coefficient of 98% (Table 4). The remaining 15 strains are distributed among 14 RFLP groups. In the majority of cases there is no obvious correlation between RFLP patterns and host of origin. For example, RFLP groups 11 and 16 include strains isolated from seven different hosts (Table 1). However, the two mulberry isolates (RFLP groups 19 and 20) and the three ginger isolates (RFLP groups 21, 22, and 23) have RFLP patterns that may represent distinct host-associated subdivisions within division I.

In contrast to division I, strains within division II can be distinguished according to both race and biovar. Although several of the probes fail to distinguish between the various strains, thus indicating their relatedness (Table 3), other probes separate division II into five distinct subdivisions (Fig. 2). Similarity coefficient calculations for subdivisions of division II are given in Table 5. The strains of race 1

Table 2. Plasmids used in this study

Plasmid	Strain	Size of *P. solanacearum* DNA insert	Tn*5* affected phenotype	Source
pKD600	K60	12.7 kb	HR−/vir−	P. Xu
pKD700	K60	8.5 kb	EPS−	P. Xu
pKD1358	K60	7.0 kb	HR−/vir−	P. Xu
pK1506	K60	8.4 kb	HR−/vir−	P. Xu
pKD1602	K60	10.1 kb	HR−/vir−	P. Xu
pT13	K2R	12.4 kb	HR−/vir−	P. Traynor
pT34	B1	7.5 kb	HR−/vir−	P. Traynor
pT140	K2R	17.0 kb	HR−/vir−	P. Traynor
pT161	K2R	3.8 kb	Tryptophan auxotroph	P. Traynor

Table 3. Summary of RFLP patterns for RFLP groups of *P. solanacearum* when analyzed with nine different probes

RFLP group	Division	Plasmid probe[a]								
		13	161	34	700	600	1358	1602	1506	140
1	II	1	1A	1	1A	1	1	1	1A	1A
2	II	1	1A	1	1A	2	1	1	1A	1A
3	II	1	1A	1	1A	2	1	1	1B	1B
4	II	1	1A	1	1A	1A	1	1	1B	1A
5	II	1	1A	1	1A	1B	1	1	1B	1B
6	II	4	1A	1	1A	1	1	1	1B	1B
7	II	NH[b]	1A	1	1A	1	1	NH	4	1B
8	I	2	2	2C	1B	3A	2	2	3	3
9	I	2	2	2A	1B	3A	6	2	3	3
10	I	2	2	2B	1B	3B	2	2	3	3
11	I	2	2	2C	1B	3B	2	2	3	4
12	I	2	2	2B	1B	3C	2	2	3	3
13	I	2	2	2C	1B	3A	2A	2	3	3
14	I	NH	2	2C	1B	3A	2A	NH	NH	3
15	I	2	2	2B	1B	3A	2	2	3	4
16	I	2	2	2C	1B	3D	2	2	3	4
17	I	2	2	2D	1B	3F	2	2	3	4
18	I	2	2	2E	1B	3B	2	2	3	4
19	I	7	2	3	1B	3B	2B	2	3	4
20	I	NH	2	3	1B	3E	2B	NH	NH	4
21	I	2	2	2C	1B	3A	2	2	3	5
22	I	2	2	2G	1B	3A	2	2	3	5
23	I	2	2	2A	1B	3A	2	2	3	5
24	II	3	1B	1	1A	1	3	3	5	6
25	II	4	1A	1	1A	1	4	3	1B	6
26	II	6	1B	1	1A	4	3	3	5	6
27	II	6	1B	1	1A	4	3	3	6	6
28	II	5	1A	1	1A	1	1	1	1B	7

[a] Numbers represent distinct RFLP patterns with respect to a single probe. Letters designate RFLP patterns that have some restriction fragments in common, but are not identical.
[b] NH, no homology.

(biovar 1) and race 3 (biovar 2) represent two relatively homogeneous subdivisions, whereas strains of race 2 form three separate subdivisions, each composed of a single RFLP group. With the exception of race 3 strains (RFLP groups 26 and 27), all isolated from potato, there is no apparent correlation between host range and RFLP patterns within division II. There is, however, a strong correlation between geographical origin and RFLP patterns within race 2. Race 2 strains isolated from Central America, from Colombia and Peru, and from Venezuela correspond to RFLP groups 24, 25, and 28, respectively (Table 1). This contrasts with results obtained for race 3 strains and, to a lesser extent, race 1 strains, which are homogeneous according to RFLP patterns but were isolated from widely different locations (Table 1).

In most instances, the RFLP data confirmed the assignment of strains to one of the three major races. However, two strains previously classified as race 1, UW 28 and K2R (unpublished; Morales *et al.* 1985), had RFLP patterns typical of race 2. To resolve this problem, pathogenicity tests on triploid banana plants (*Musa* AAA), which are susceptible to race 2 but not to race 1 strains, were completed. Both UW 28 and K2R were highly pathogenic to triploid banana, indicating that, as predicted by the RFLP data, they belong to race 2. The fact that both strains cause the HR on tobacco leaves, as is typical of all race 2 strains, provides further support for this conclusion (Lozano and Sequeira 1970). In a previous abstract, K2R was listed as a spontaneous mutant of the race 1 strain UW 25 (K60) (Morales *et al.* 1985); this was incorrect.

DISCUSSION

The RFLP analysis of 62 strains of *P. solanacearum* distinguished 28 unique groups. Similarity coefficients allowed separation of the species into two major divisions: division I contains all members of race 1 biovars 3 and 4 and the two race 1 biovar 5 strains from mulberry, whereas division II contains all race 1 biovar 1 strains and races 2 and 3. The separation of the species into these two major divisions is not indicated by either of the current methods of classification and differs sharply from the race system of classification that, for example, places the widely different members of biovar 1 and biovars 3 and 4 in a single race.

On the basis of analyses of DNA homologies and physiological characteristics of strains of *P. solanacearum*, Palleroni and Doudoroff (1971) and Harris (1972) could find no obvious differences between biovars 3 and 4 and suggested that these strains might form a distinct group within *P. solanacearum*. Our results confirm this conclusion. In studies with other biovars, Harris (1972) concluded that biovar 2 was similar to biovar 1. This is additional support for the conclusion that biovar 2 and biovar 1 strains are members of a relatively homogenous group.

The strains used in this study were collected by several investigators over the past three decades and do not represent a methodical attempt to generate a collection of strains representative of this species. However, in selecting the 62 isolates used, an attempt was made to represent the broad host range and wide geographic distribution of *P. solanacearum*. Of the 38 strains of race 1, 12 had been isolated from separate host species in 11 different countries. Race 2 strains were isolated from three different hosts in five countries in South and Central America, and representatives of race 3 were obtained from infected potato plants in four countries.

We expected that the probes encoding functions involved

Table 4. Similarity coefficients for 28 RFLP groups of *Pseudomonas solanacearum*[a]

	RFLP groups																									
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
1	**95**	**85**	**80**	**80**	**78**	**65**	18	17	18	17	18	17	10	17	13	17	17	17	9	16	16	16	**42**	**65**	**40**	**38**	**70**
2		**68**	**74**	**68**	**62**	**46**	14	14	14	13	14	14	5	13	14	13	13	13	5	13	13	13	**44**	**47**	**33**	**31**	**53**
3			**85**	**95**	**93**	**76**	13	13	13	13	13	13	10	13	9	13	13	13	9	12	12	12	**47**	**75**	**44**	**43**	**85**
4				**85**	**78**	**59**	13	13	13	13	13	13	10	13	13	13	13	13	9	12	12	12	**37**	**65**	**44**	**43**	**70**
5					**88**	**86**	13	13	13	13	13	13	10	13	9	13	13	13	9	12	12	12	**42**	**70**	**44**	**43**	**80**
6						**74**	13	13	13	13	13	13	10	13	13	13	13	13	9	12	12	12	**45**	**83**	**48**	**46**	**83**
7							10	9	10	9	10	9	11	9	5	9	9	9	10	9	9	9	**50**	**59**	**38**	**36**	**70**
8								**86**	**84**	**77**	**88**	**98**	**71**	**85**	**78**	**81**	**73**	**58**	**49**	**85**	**84**	**81**	13	18	12	12	13
9									**75**	**64**	**78**	**85**	**70**	**75**	**65**	**72**	**64**	**53**	**44**	**73**	**75**	**76**	12	17	12	11	13
10										**85**	**92**	**82**	**67**	**77**	**78**	**69**	**85**	**69**	**45**	**70**	**69**	**70**	13	18	12	12	13
11											**73**	**75**	**60**	**81**	**98**	**74**	**96**	**81**	**59**	**79**	**77**	**75**	8	13	8	7	17
12												**86**	**62**	**81**	**75**	**73**	**73**	**58**	**49**	**74**	**73**	**74**	13	18	12	12	13
13													**87**	**83**	**77**	**79**	**72**	**57**	**48**	**84**	**82**	**80**	12	17	12	11	13
14														**68**	**61**	**64**	**55**	**38**	**55**	**69**	**68**	**65**	14	15	13	13	10
15															**87**	**93**	**85**	**70**	**63**	**86**	**84**	**86**	8	13	8	7	17
16																**83**	**91**	**75**	**60**	**80**	**79**	**76**	4	9	4	4	13
17																	**81**	**67**	**59**	**82**	**81**	**82**	8	13	8	7	17
18																		**81**	**59**	**75**	**74**	**75**	8	13	8	7	17
19																			**67**	**61**	**60**	**61**	8	13	8	7	17
20																				**60**	**59**	**60**	9	9	8	8	14
21																					**98**	**97**	8	12	7	7	16
22																						**98**	8	12	7	7	16
23																							8	12	7	7	16
24																								**56**	**75**	**64**	**47**
25																									**58**	**55**	**70**
26																										**88**	**40**
27																											**38**

[a] Similarity coefficient: S = (2Nxy) (100) / (Nx + Ny). Values in bold represent similarity coefficients calculated within division I or division II.

in host-pathogen interaction might be better than randomly selected probes in distinguishing strains with different pathogenic specializations. This, however, was not the case; for example, probes affecting tryptophan and EPS biosynthesis, pT161 and PK700, respectively, revealed the same major trends as did the probes that specify virulence and HR. The fact that certain probes accounted for much of the observed diversity may indicate that the apparent taxonomic relationships are an artifact of the particular probes used in the analysis. However, taken as a whole, the data reflected the groupings obtained by traditional methods of classification. Thus, a meaningful estimate of taxonomic relatedness does not appear to depend so much on the nature of the probes as on the number of probes that are used in the analysis. The fact that we were able to use RFLP analysis to determine accurately the race and biovar of two incorrectly classified isolates, K2R and UW 28, suggests that the nine probes we used were adequate for

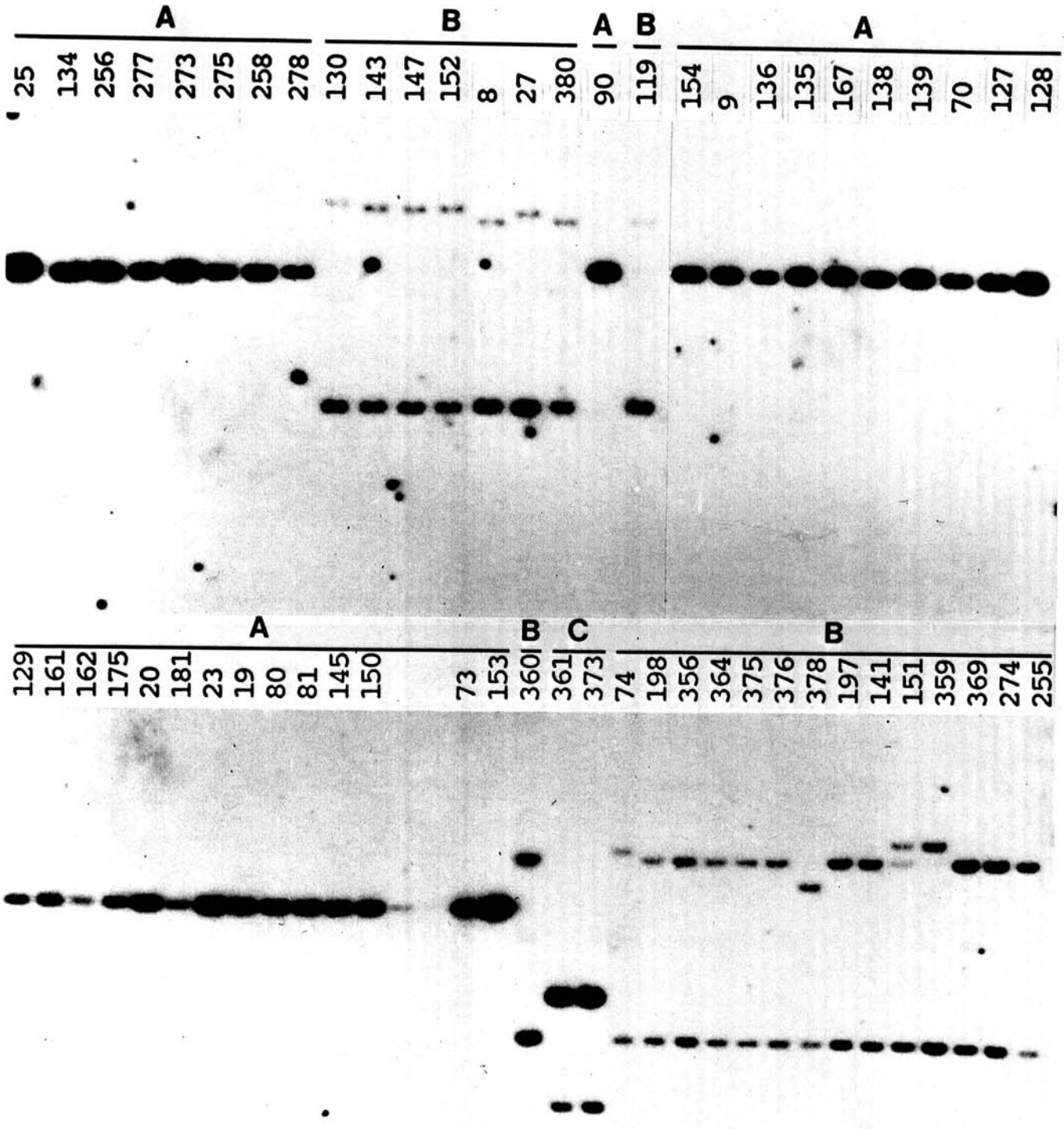

Fig. 1. Southern blot analysis by probe pT34 of *Eco*RI-digested total genomic DNA from 58 strains of *Pseudomonas solanacearum*. **A**, division II strains; **B**, division I strains, except C; **C**, division I mulberry strains.

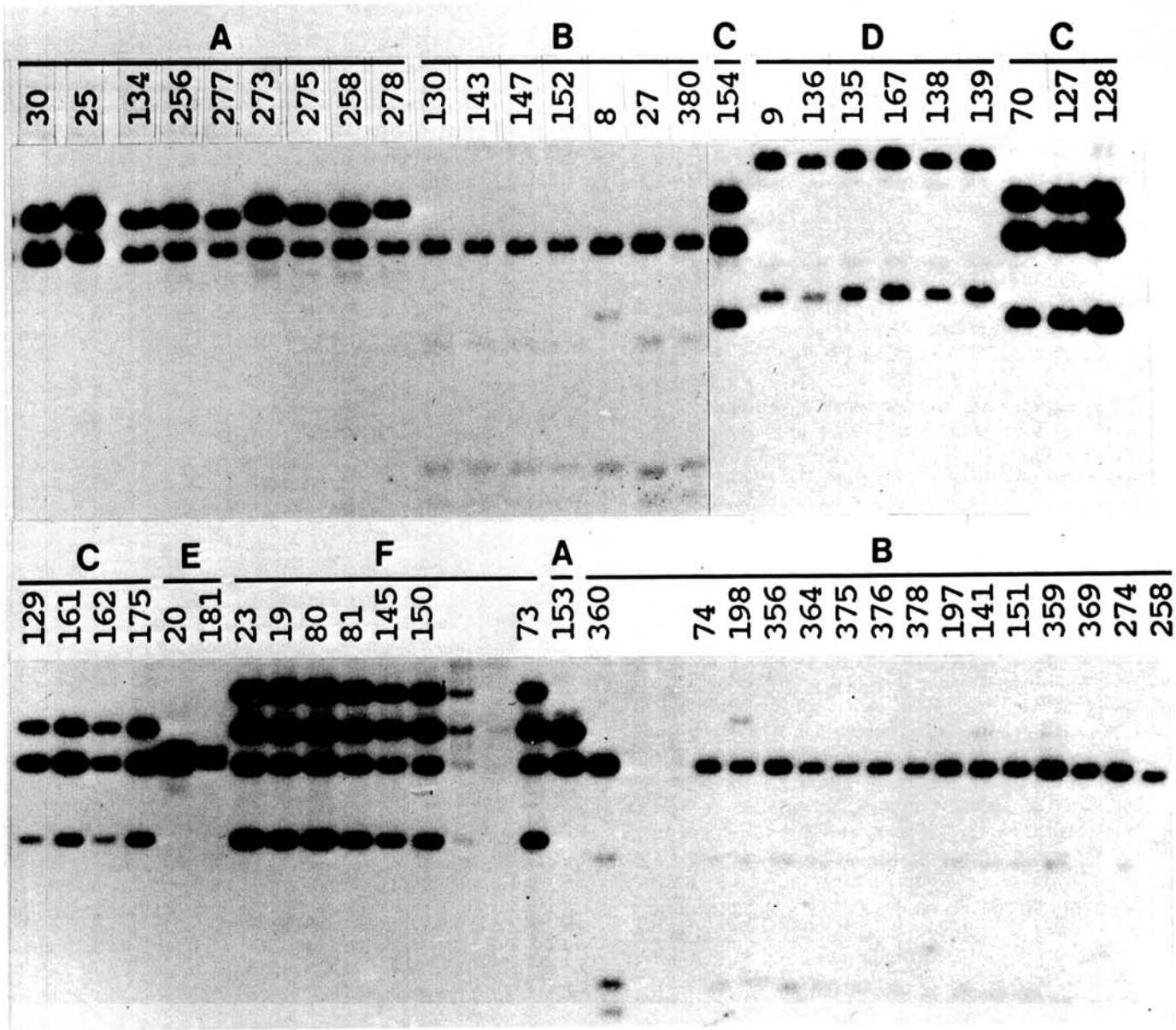

Fig. 2. Southern blot analysis by probe pT13 of *Eco*RI and *Bam*HI-digested total genomic DNA from 55 strains of *Pseudomonas solanacearum*. **A**, race 1 biovar 1; **B**, race 1 biovars 3 and 4; **C**, race 2, RFLP group 25; **D**, race 2, RFLP group 24; **E**, race 2, RFLP group 28; **F**, race 3. When probed with pT13, strain 154 produces a pattern characteristic of race 2, RFLP group 25. However, in the overall analysis (Tables 1 and 3) we have classified it as race 1 biovar 1.

Table 5. Similarity coefficients for the 12 RFLP groups within division II[a]

	RFLP Groups[b]				
	1–7	**24**	**25**	**28**	**26, 27**
1-7	77[c]	43	68	73	41
24		100	56	47	70
25			100	70	57
28				100	39
26, 27					88

[a] Similarity coefficient: $S = (2Nxy)(100)/(Nx + Ny)$.
[b] RFLP groups 1–7, race 1 biovar 1; 24, race 2; 25, race 2; 28 race 2; 26 and 27, race 3.
[c] When RFLP group 7, which lacks homology with probe pT13, is omitted from the calculation, then S = 81.

determining general taxonomic relationships. The numerical estimates of similarity, however, would probably be improved by use of additional probes.

One of the most intriguing questions regarding the taxonomy of *P. solanacearum* concerns the evolutionary relationships and geographical origin of members within the species. The RFLP data suggest that the species evolved as two separate groups of strains, perhaps as the result of geographical isolation. With respect to division II, it is probable that races 2 and 3 originated in Central and South America. Members of race 2, for example, had not been found outside of this area until infected banana rhizomes were moved from Central America to the Philippines (Buddenhagen 1985). Similarly, race 3 is endemic in the Andean regions of South America, where potatoes originated, and is a homogeneous group of strains that is almost entirely restricted to potatoes. The high degree of similarity in RFLP patterns among the race 3 strains that we examined, although they came from many widely separated parts of the world, supports Buddenhagen's contention that

the occurrence of race 3 outside of South America is the result of the distribution of infected potato tubers by humans.

Because RFLP analyses indicate that race 1 biovar 1 is closely related to both races 2 and 3, we have concluded that all members of division II may have originated in the Americas. This is supported by the isolation of strains of race 1 (biovar 1) from plant hosts in virgin areas of Central America (Buddenhagen 1960) and the conspicuous absence of race 1 biovar 1 strains from many areas of Asia (Buddenhagen 1985; He *et al.* 1983).

The usefulness of RFLP patterns not only as indicators of relatedness of strains, but of the evolution of the species as geographically distinct clonal populations is exemplified by the race 2 strains included in RFLP groups 24, 25, and 28. The relatively recent epidemics of bacterial wilt of banana in South and Central America apparently resulted from dissemination of several distinct strains of *P. solanacearum* (French and Sequeira 1970). Consistent with this conclusion, we identified three dissimilar RFLP groups within race 2, each of which was associated with a different epidemic of unique geographic origin.

Another point of interest concerns the host range of *P. solanacearum*. Races 2 and 3 have relatively narrow host ranges, being restricted primarily to musaceous hosts and potatoes, respectively. These strains have easily recognizable RFLP patterns. On the other hand, race 1 is known to possess a wide host range, and this diversity is reflected in the results of RFLP analysis. Although we searched for host-associated groups within race 1, with the exception of two mulberry strains (UW 361 and 373) and three ginger strains (UW 141, 151, and 359), these groups were not obvious. Of 10 multiple-strain RFLP groups, seven contain strains from different hosts. For instance, RFLP group 11 contains six strains from five different hosts. The ability of individual strains of *P. solanacearum* to infect a wide range of hosts is well known, but is based on greenhouse inoculations that may not provide a true indication of the pathogenicity of these strains in the field (Pegg and Moffett 1971; French and Sequeira 1970). The present RFLP analysis, however, confirms that isolations from diverse hosts in the field may yield similar or perhaps identical strains.

Two new races of *P. solanacearum* have been proposed for strains with host range limited to mulberry (He *et al.* 1983) and ginger (Hayward *et al.* 1967; Pegg and Moffett 1971). In this study two groups of strains within division I, composed exclusively of strains from mulberry (UW 361 and 373) and ginger (UW 141, 151, and 359), are unique with respect to several probes. It is known that the two mulberry strains (UW 361 and 373) have a limited host range (He *et al.* 1983). This correlation indicates that strains with restricted host range may possess unique RFLP patterns.

In three instances, strains that failed to show homology with the probes pT13, pK1602, and pK1506 were identified (Table 3). In a separate study, we have determined that these *Eco*RI fragments are part of a cluster where several *hrp* mutations are located. Part of this cluster shares homology, but not at the level of restriction fragment lengths, with the clustered *hrp* genes reported by Boucher *et al.* (1987). Boucher (1988) determined that these genes were deleted from acridine orange-resistant cells. Our data demonstrate that these genes are subject to deletion at a high frequency (three out of 62 strains tested) in the absence of acridine orange; however, the origin of these deletions is uncertain.

These initial results indicate that RFLP analysis offers an objective means to reevaluate the classification of strains of *P. solanacearum*. With this technique, strains can be classified according to similarity of RFLP patterns (expressed quantitatively), and these results may be compared with traditional taxonomic groupings. The fact that RFLP data was used to predict accurately the race of two incorrectly classified strains, UW 28 and K2R, demonstrates the utility and strength of this technique. Our work involved a limited number of strains, but it indicates that a revised classification for the species, based on RFLP analysis, is possible. When the ongoing analysis of many additional strains is completed, we believe that a classification that reflects the natural evolution of the species will be possible.

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LITERATURE CITED

Amasino, R. M. 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. Anal. Biochem. 152:304-307.

Boucher, C. A., Barberis, P. A., and Arlat, M. 1988. Acridine orange selects for deletion of *hrp* genes in all races of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 1:282-288.

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. 169:5626-5632.

Buddenhagen, I. 1960. Strains of *Pseudomonas solanacearum* in indigenous hosts in banana plantations of Costa Rica, and their relationship to bacterial wilt of bananas. Phytopathology 50:660-664.

Buddenhagen, I., Sequeira, L., and Kelman, A. 1962. Designation of races in *Pseudomonas solanacearum*. Phytopathology 52:726.

Buddenhagen, I. 1985. Bacterial wilt revisited. Pages 126-143 in: Bacterial Wilt Disease in Asia and the South Pacific, No. 13. G. J. Persley, ed. ACIAR, Canberra, Australia.

French, E., and Sequeira, L. 1970. Strains of *Pseudomonas solanacearum* from Central and South America: A comparative study. Phytopathology 60:506-512.

Gabriel, D. W., Hunter, J. E., Kingsley, M. T., Miller, J. W., and Lazo, G. R. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. 1:59-65.

Harris, D. 1972. Intra-specific variation in *Pseudomonas solanacearum*. Pages 289-292 in: Proc. 3rd Int. Conf. Plant Path. Bact. Wagenigen, The Netherlands.

Hayward, A. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bact. 27:265-277.

Hayward, A. C., Moffett, M. C., and Pegg, K. G. 1967. Bacterial wilt of ginger in Queensland. Queensl. J. Agric. Anim. Sci. 24:1-5.

He, L., Sequeira, L., and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 67:1357-1361.

Hendrick, C. A., and Sequeira, L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. 48:94-101.

Kelman, A. 1953. The Bacterial Wilt Caused by *Pseudomonas solanacearum*. A Literature Review and Bibliography. N.C. Agric. Exp. Stn. Tech. Bull. 99.

Kelman, A. 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. A gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Lozano, J., and Sequeira, L. 1970. Differentiation of races of *Pseudomonas solanacearum* by a leaf infiltration technique. Phytopathology 60:833-838.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morales, V. M., Stemmer, W. P. C., and Sequeira, L. 1985. Genetics of avirulence in *Pseudomonas solanacearum*. Pages 89-96 in: Plant Cell/Cell Interactions. I. Sussex, A. Ellingboe, M. Crouch, and R. Malmberg, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morton, D., Dukes, P., and Jenkins, S. 1966. Serological relationships of races 1, 2 and 3 of *Pseudomonas solanacearum*. Plant Dis. Rep. 50:275-277.

Nei, M., and Li, W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269-5273.

Palleroni, N., and Doudoroff, M. 1971. Phenotypic characterization and deoxyribonucleic acid homologies of *Pseudomonas solanacearum*. J. Bacteriol. 107:690-696.

Pegg, K., and Moffett, M. 1971. Host range of the ginger strain of *Pseudomonas solanacearum* in Queensland. Aust. J. Exp. Agric. Anim. Husb. 11:696-698.

Seneviratne, A. 1969. On the occurrence of *Pseudomonas solanacearum* in the hill country of Ceylon. J. Hortic. Sci. 44:393-402.

Sequeira, L., and Averre, C. 1961. Distribution and pathogenicity of strains of *Pseudomonas solanacearum* from virgin soils in Costa Rica. Plant Dis. Rep. 45:435-440.

Tabei, H., and Quimio, A. J. 1978. Strain differentiation of *Pseudomonas solanacearum* affecting solanaceous crops in the Philippines. Jap. Agric. Res. Q. 12:238-240.

Xu, P., Leong, S., and Sequeira, L. 1988. Molecular cloning of genes that specify virulence in *Pseudomonas solanacearum*. J. Bacteriol. 170:617-622.