APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2003, p. 2936–2941 Vol. 69, No. 5
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.5.2936–2941.2003

Identification of an Emergent and Atypical *Pseudomonas viridiflava* Lineage Causing Bacteriosis in Plants of Agronomic Importance in a Spanish Region

Ana J. González,[1] M. Rosario Rodicio,[2] and M. Carmen Mendoza[2]*

Servicio Regional de Investigación y Desarrollo Agroalimentario, Principado de Asturias, Villaviciosa,[1] and Departamento de Biología Funcional, Area Microbiología, Universidad de Oviedo, Oviedo,[2] Spain

Received 6 December 2002/Accepted 24 February 2003

***Pseudomonas* strains with an atypical LOPAT profile (where LOPAT is a series of determinative tests: L, levan production; O, oxidase production; P, pectinolitic activity; A, arginine dihydrolase production; and T, tobacco hypersensibility) can be regarded as emergent pathogens in the Principality of Asturias (Spain), where they have been causing, since 1999, severe damage in at least three taxonomically unrelated orchard plants of agronomic importance: common bean (*Phaseolus vulgaris*), kiwifruit (*Actinidia deliciosa*), and lettuce (*Lactuca sativa*). These strains are mainly differentiated by production of yellowish mucoid material in hypersucrose medium, used for the levan test, and by a variable pectinolytic activity on different potato varieties. The atypical organisms were identified as *Pseudomonas viridiflava* based on their 16S rRNA sequences. Among them a certain intraspecies genetic heterogeneity was detected by randomly amplified polymorphic DNA (RAPD) typing. To differentiate between isolates of *P. viridiflava* and *Pseudomonas syringae* pathovars, a 16S ribosomal DNA restriction fragment length polymorphism method employing the restriction endonucleases *Sac*I and *Hin*fI was developed. This could be used as a means of reliable species determination after the usual phenotypical characterization, which includes the LOPAT tests.**

The phytopathogenic oxidase-negative fluorescent *Pseudomonas* species have been traditionally grouped into two species, *P. syringae* and *P. viridiflava* (17), the former including more than 40 well-characterized pathovars. The term pathovar refers to strains grouped at the subspecies level on the basis of plant host range and symptoms and with the aid of biochemical tests (8, 25). Of these, the LOPAT determinative tests (L, levan production; O, oxidase production; P, pectinolytic activity; A, arginine dihydrolase production; and T, tobacco hypersensibility) (10) is widely applied to differentiate isolates. Although of practical interest, bacterial phenotypical characterization alone often fails to reveal genetic relationships within or between species. In fact, DNA hybridization analyses quite early revealed that *P. syringae* is a heterogeneous species (18), which is still subject to extensive revision (6, 21).

The Principality of Asturias (PA) is a region on the north coast of Spain in which agriculture is mainly centered in orchard plants and fruit trees. In the Phytopathology Laboratory of the Regional Service of Agrofood Research and Development of the PA (LPPA), *P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola* (also named *P. savastanoi* pv. *phaseolicola* or *P. amygdali*) (16) have been identified as pathogens of plants with high agronomic value. Of these, *P. syringae* pv. *syringae* was found to be the pathovar displaying a wider host range, causing severe bacteriosis in practically all orchard plants under culture in Asturias as well as in fruit trees, whereas *P. syringae* pv. *phaseolicola* has been only collected from common bean plants with disease symptoms (7). *P. viridiflava*, rarely found in the PA, has been traditionally considered to be an epiphyte or opportunistic pathogen (11, 2). Since 1999, a new *Pseudomonas* type with an atypical LOPAT profile (convex colonies with uncharacteristic yellowish mucoid material in hypersucrose medium [L test]; O negative; P variable; A negative; and T positive) has been frequently isolated from and associated with disease in common bean plants. Later, it has also appeared in material from other plants with disease symptoms, including kiwifruits (from spring of 2000) and lettuce (from 2001). The aim of the present study was to identify the phytopathogenic *Pseudomonas* with the atypical LOPAT profile which can be regarded as emergent in the PA. The species was identified by sequencing of the DNA encoding 16S rRNA as *P. viridiflava*, the pathogenicity was verified according to Koch's postulates, and the genetic types causing disease were traced by randomly amplified polymorphic DNA (RAPD) segment analysis. In addition, a genetic procedure based on restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA genes was developed to differentiate *P. viridiflava* from *P. syringae* isolates.

MATERIALS AND METHODS

Bacterial strains. In this study 31 isolates belonging to the fluorescent *Pseudomonas* group and displaying an atypical LOPAT profile were analyzed. They were collected from common bean (19), kiwifruits (10), and lettuce (2) plants with disease symptoms from different orchard sites of the PA (Table 1). The PA is a coastal Cantabrian region with a wet and mild climate where the three mentioned species are important crops. In addition, other bacteria were included as reference or outgroup strains, including seven strains previously identified in the LPPA as *P. viridiflava*, *P. syringae* pv. *syringae*, or *P. syringae* pv. *phaseolicola* (one, three, and three strains, respectively), and three collection strains: *P. viridiflava* CECT 458 (ATCC 13223), *P. syringae* pv. *syringae* CECT 4429 (ATCC 19310), and *P. syringae* pv. *phaseolicola* CECT 321 (ATCC 19304).

Isolation procedure and phenotypic characterization of *Pseudomonas* strains.

* Corresponding author. Mailing address: Area de Microbiología. Facultad de Medicina, C/Julián Clavería 6, 33006 Oviedo, Spain. Phone: 34 985103560. Fax: 34 985103148. E-mail: cmendoza@correo.uniovi.es.

TABLE 1. Origin and relevant features of *Pseudomonas* strains analyzed in this work

Strain/yr	Sample origin			Result[d]		16S rDNA RFLP		RAPD pattern	Species
	Bost	Plant sample	Orchard locality	L test	P test	*Sac*I	*Hin*fI		
LPPA 74/99	Bean	Seed	Navia	+ [y]	+	S-1	H-2	R-2	*P. viridiflava*
LPPA 76/99	Bean	Seed	Navia	+ [y]	−	S-1	H-2	R-2	*P. viridiflava*
LPPA 77/99	Bean	Seed	Navia	+ [y]	−	S-1	H-2	R-2	*P. viridiflava*
LPPA 78/99	Bean	Seed	Navia	+ [y-g]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 80/99	Bean	Seed	Navia	+ [y-g]	−	S-1	H-2	R-2	*P. viridiflava*
LPPA 81/99	Bean	Pod	Tapia	+ [y]	v	S-1	H-2	R-2	*P. viridiflava*
LPPA 83/00	Bean	Pod	Villaviciosa	+ [y-g]	v	S-1	H-2	R-1	*P. viridiflava*
LPPA 84/00[a,b,c]	Bean	Pod	Villaviciosa	v	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 88/00	Bean	Stem	Vegadeo	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 89/01	Bean	Seed	Valdés	+ [y-g]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 91/01	Bean	Seed	Valdés	+ [y]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 94/01	Bean	Seed	Vegadeo	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 249/01	Bean	Pod	Tineo	+ [y]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 252/01	Bean	Pod	Tineo	+ [y]	+	S-1	H-2	R-2	*P. viridiflava*
LPPA 280/01	Bean	Seed	Pravia	+ [y-g]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 287/02	Bean	Seed	Valdés	+ [y]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 288/02[a,b]	Bean	Seed	Valdés	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 290/02	Bean	Seed	Valdés	+ [y]	+	S-1	H-2	R-6	*P. viridiflava*
LPPA 319/02	Bean	Leaf	Villaviciosa	+ [y]	−	S-1	H-2	R-1	*P. viridiflava*
LPPA 102/00	Kiwifruit	Leaf	Villaviciosa	+ [y]	−	S-1	H-2	R-3	*P. viridiflava*
LPPA 103/00	Kiwifruit	Leaf	Villaviciosa	+ [y]	+	S-1	H-2	R-3	*P. viridiflava*
LPPA 118/00	Kiwifruit	Leaf	Villaviciosa	+ [y]	−	S-1	H-2	R-4	*P. viridiflava*
LPPA 125/00	Kiwifruit	Leaf	Villaviciosa	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 126/00	Kiwifruit	Leaf	Villaviciosa	+ [y]	+	S-1	H-2	R-5	*P. viridiflava*
LPPA 139/00[a,b]	Kiwifruit	Bud	Villaviciosa	+ [y]	v	S-1	H-2	R-1	*P. viridiflava*
LPPA 143/00[a,b]	Kiwifruit	Bud	Grado	+ [y]	v	S-1	H-2	R-2	*P. viridiflava*
LPPA 144/00[a,b]	Kiwifruit	Bud	Pravia	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 147/00	Kiwifruit	Bud	Llanes	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 150/00	Kiwifruit	Leaf	Llanes	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 264/02[a,b]	Lettuce	Leaf	Villaviciosa	+ [y]	+	S-1	H-2	R-1	*P. viridiflava*
LPPA 265/02[a,b]	Lettuce	Leaf	Villaviciosa	+ [y]	+	S-1	H-2	R-2	*P. viridiflava*
Control strains									
CECT 458[a,b]				−	++	S-1	H-2	R-1	*P. viridiflava*
CECT 4429[a]				+ [w]	−	S-2	H-1	R-10	*P. syringae* pv. *syringae*
CECT 321[a]				+ [w]	−	S-2	H-1	R-20	*P. syringae* pv. *phaseolicola*
LPPA 79/99	Bean	Seed	Navia	−	+	S-1	H-2	R-2	*P. viridiflava*
LPPA 86/00	Bean	Pod	Vegadeo	+ [w]	−	S-2	H-1	R-11	*P. syringae* pv. *syringae*
LPPA 93/01	Bean	Seed	Vegadeo	+ [w]	−	S-2	H-1	R-12	*P. syringae* pv. *syringae*
LPPA 136/00	Kiwifruit	Leaf	Villaviciosa	+ [w]	−	S-2	H-1	R-13	*P. syringae* pv. *syringae*
LPPA 87/00	Bean	Pod	Valdés	+ [w]	−	S-2	H-1	R-20	*P. syringae* pv. *phaseolicola*
LPPA 244/01	Bean	Pod	Valdés	+ [w]	−	S-2	H-1	R-20	*P. syringae* pv. *phaseolicola*
LPPA 245/01	Bean	Pod	Villayón	+ [w]	−	S-2	H-1	R-20	*P. syringae* pv. *phaseolicola*

[a] Strain tested for pathogenicity.
[b] Strain whose 16S rDNA has been sequenced.
[c] Polysaccharide-positive and-negative variants were tested for patogenicity.
[d] Abbreviations and symbols: y, yellow; y-g, yellow-greenish; w, white; −, negative for the indicated feature; +, positive for the indicated feature; ++, strong pectinolytic activity; v, variable result.

Samples of seeds, stems, leaves, and pods from beans; floral buds from kiwifruits; and lettuce leaves with disease symptoms, collected at different times over the period 1999 to 2001, from different orchard sites (Table 1), were analyzed for bacteriosis. The bacterial isolation procedure on King's medium B was used (9). Fluorescent bacteria obtained on this medium were tested for biochemical traits, including Hugh-Leifson reaction; LOPAT profile; sculine and gelatin hydrolysis; and mannitol, erythritol, sorbitol, *m*-inositol, adonitol, betaine, homoserine, trigonelline, D-tartrate, and quinate assimilation in Ayers's minimal medium (16). Stability of the LOPAT profile was tested after successive subcultures in King's medium B. Isolates showing identical features, and collected from the same orchard site at the same period of the year, were considered to be a single strain.

Pathogenicity tests. The virulence of different *Pseudomonas* strains, collected from different host plants and showing the atypical LOPAT pattern (Table 1), were tested for Koch's postulates, using the plant from which they were originally collected as the host. The inoculations were performed by spraying bacterial suspensions (10^6 and/or 10^9 CFU/ml) on sets of bean and lettuce plantules (10 plantules/set), or 6 to 10 buds of kiwifruit. *P. syringae* pv. *syringae* LPPA 86, *P. syringae* pv. *phaseolicola* LPPA 87, and *P. viridiflava* CECT 458 were tested in the same way. Beans and lettuce plant sets were maintained at 22°C with a light period of 16 h/day, covered with a transparent plastic bag during the initial 48 h. Kiwifruit buds in the field were also kept enclosed in plastic bags for 48 h. Assays were repeated at least twice. When disease symptoms appeared, part of the damaged tissues was processed and aliquots were plated onto King's medium B. After incubation (48 h at 25°C) fluorescent bacteria were recovered and subjected to biochemical and genetic tests (see below). All inoculated material was autoclaved before being discarded.

PCR amplification of 16S ribosomal DNA (rDNA). Almost-full-length 16S rRNA genes were amplified from all the isolates with an atypical LOPAT pattern and control strains compiled in Table 1, using the pA and pH′ primers designed by Edwards et al. (5) for eubacteria. Amplifications were done in a MJ Research (Waltham, Mass.) PTC 100 PCR system, using 50-μl volumes that contained 1 μl of DNA extracted according to the method of Deener and Boychuck (3), 0.3 μM concentrations of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, 2 U of DyNAZyme II DNA polymerase (Finnzymes Oy, Espoo,

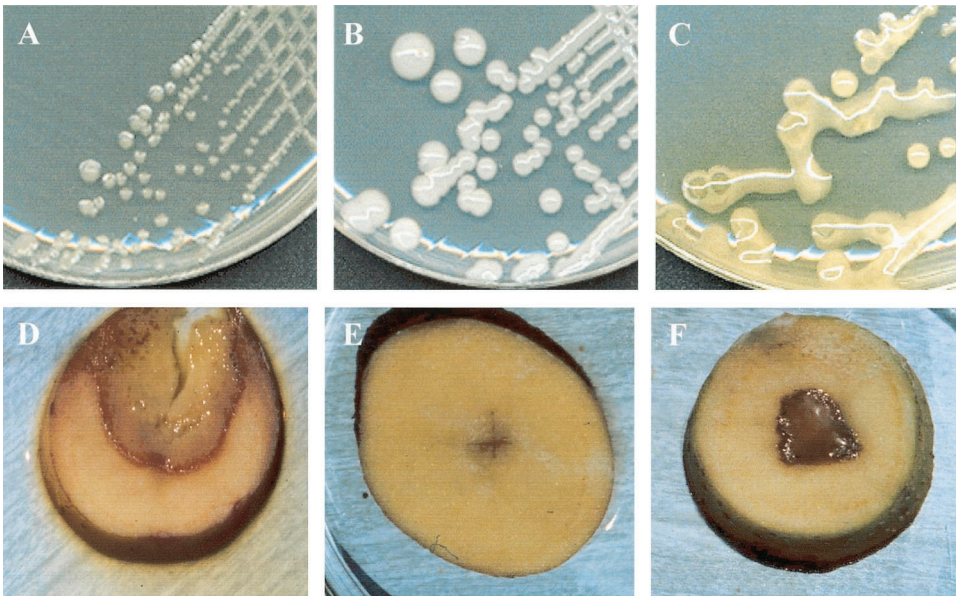

FIG. 1. Differential LOPAT tests of atypical *Pseudomonas* isolates. Growth on hypersucrose medium (L test) (A to C) and pectinolytic activity on potato slices (D to F) are shown. (A) *P. viridiflava* CECT 458 (negative control); (B) *P. syringae* pv. *syringae* CECT 4429 (positive control); (C) atypical *P. viridiflava* LPPA 144; (D) *P. viridiflava* CECT 458 (positive control); (E) *P. syringae* pv. *syringae* CECT 4429 (negative control); (F) atypical *P. viridiflava* LPPA 144.

Finland), and 5 μl of the supplier's reaction buffer. Volume was made up to 50 μl with sterile double-distilled water. After a 3-min denaturation step at 94°C, the reaction mixture was run through 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 90 s, followed by a final step at 72°C during 10 min. The ca. 1,500-bp PCR products were purified with the Ultraclean PCR clean up DNA purification kit (MO-BIO, Inc.), as recommended by the manufacturer, and used directly for sequencing analysis and/or for RFLP profiling (see below).

16S rDNA sequencing and sequence analysis. Selected PCR fragments, amplified from the isolates tested for pathogenicity and from the control strain *P. viridiflava* CECT 458, were sequenced in both strands, with the pA and pH′ primers by the Servicio de Secuenciación de DNA, Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas, Madrid, Spain, using the BigDye Terminator cycle sequencing ready reaction FS kit and an ABI PRISM 3700 DNA sequencer (both from Applied Biosystems Div., Foster City, Calif.). To determine their phylogenetic affiliation, sequences were initially compared to the available databases by using BLAST (1). Phylogenetic trees were generated by neighbor joining, using Jukes-Cantor corrected distances, within the RDP-II PHYLIP interface (12). The accession numbers of the 16S rDNA sequences used for comparison were as follows: *P. aeruginosa* LMG 1242T, Z76651; *P. amygdali* LMG 2123T, Z76654; *P. cichorii* LMG 2162T, Z76658; *P. fluorescens* DSM 50090T, Z76662; *P. marginalis* LMG 2210T, Z76663; *P. syringae* pv. *actinidiae*, AB001439; *P. syringae* pv. *maculicola*, AB001444; *P. syringae* pv. *morsprunorum*, AB001445; *P. syringae* pv. *phaseolicola*, AB001448; *P. syringae* pv. *theae*, AB0001450; *P. syringae* pv. *syringae* LMG 1247 t1T, Z76669; and *P. viridiflava* LMG 2352T, Z76671 (14, 20).

RFLP and RAPD procedures. Genetic types were traced by RFLP analysis of almost-entire 16S rRNAs genes and by RAPD typing of total genomic DNA of the bacteria under study. For RFLP analysis, 16S rDNA sequences generated in this work, together with relevant *Pseudomonas* sequences retrieved from databases, were examined by using the MAP-SORT program of the University of Wisconsin Genetics Computer Group. In this way, *Sac*I and *Hin*fI restriction enzymes were selected to distinguish between species. Restriction digestions were performed on 10 μl of PCR product, with enzymes supplied by Amersham Biosciences (Barcelona, Spain), in accordance with the manufacturer's instructions. RAPD typing of genomic DNA was carried out as described by Soto et al. (22), using random primer S (TCACGATGCA) (23).

Nucleotide sequence accession numbers. Sequences generated in this work have been deposited in GenBank under accession numbers AY180967 to AY180972.

RESULTS

Phenotypic characterization of phytopathogenic *Pseudomonas* isolates with an atypical LOPAT profile. Since 1999, fluorescent bacteria with an atypical LOPAT profile (Table 1) have been isolated from plant parts of common beans (seeds, leaves, stems, and pods), lettuces (leaves), and kiwifruits (floral buds) with disease symptoms. Essentially, they were differentiated by the L test and their pectinolytic activity. They produced raised colonies with mucoid material of yellowish color (occasionally greenish) on hypersucrose medium, in contrast to the typical white mucoid material associated with levan producer strains, such as *P. syringae* pathovars (Fig. 1A to C). The mucoid material was not detected in sucrose-deficient medium. The strains showed a variable pectinolytic activity when assayed on potato slices, with rotting halos ranging from 0 up to about 10 mm surrounding two streaks inoculated in a cross pattern (Fig. 1D to F). This rotting capacity was remarkably lower than that of the control *P. viridiflava* strain. With respect to other LOPAT tests, the isolates were oxidase negative, arginine dihydrolase negative, and tobacco hypersensibility positive. However, the last of these characteristics, as well as production of the yellowish mucus, could be lost after subculture of some of the isolates in King's medium B but recovered after inoculation of the bacteria in the host plant. In contrast, the *P. syringae* pv. *syringae* strains used as controls remained levan positive after subculture. This LOPAT profile (which does not exactly fit that of any of the recognized phytopathogenic *Pseudomonas* spp.) is closest to those expected either for *P. syringae* or *P. viridiflava*. The newly isolated bacteria showed other biochemical properties in common (including oxidation of glucose in Hugh-Leifson medium, hydrolysis of esculin and

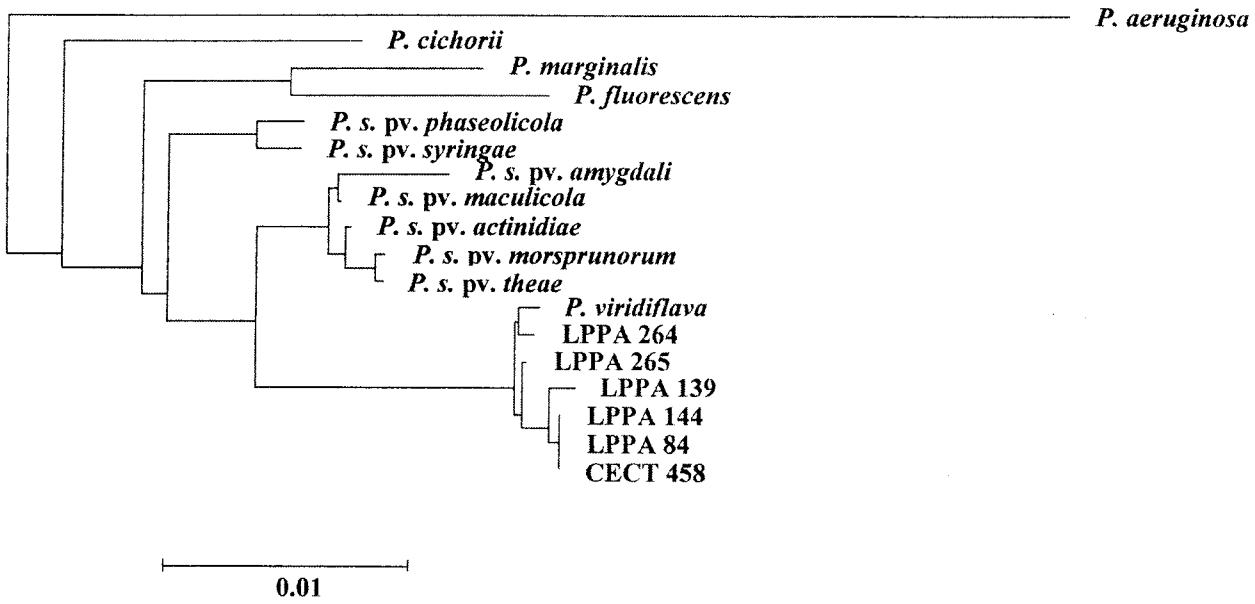

FIG. 2. Inferred phylogenetic relationships among the atypical isolates and validly described members of the genus *Pseudomonas* (sensu stricto). See Materials and Methods for accession numbers and details on the construction of the tree.

gelatin, utilization of the same set of carbon compounds), which are all shared by the above-mentioned species.

Pathogenicity studies. Symptoms observed in plants from which the atypical *Pseudomonas* strains were isolated displayed a considerable variation. In common beans, they ranged from the appearance of strikingly red spots, mainly in petioles and pods, to plant death due to systemic infection and associated to destruction of the medulla. In lettuce, soft rot of some of the leaves could also progress and result in plant death. In kiwifruits infection was characterized by the appearance of dark brown spots in floral buds that developed into extensive rot, leading to the dropping of most buds or to production of small and distorted fruits. To prove that disease symptoms were due to the atypical *Pseudomonas*, the virulence of seven isolates (two from common beans, two from lettuce, and three from kiwifruit) (Table 1) was tested through inoculation on plant sets of their original host as described in Materials and Methods. All the isolates were able to experimentally reproduce the symptoms of natural infection in their respective hosts, although those that have lost the ability to produce exopolysaccharide after subculture (LPPA 84/00) required longer. In general, the damage caused by the atypical isolates was significantly higher than that caused by the control *P. syringae* strains, whereas *P. viridiflava* CECT 458 only produced severe damage in kiwifruit floral buds, as expected.

Special attention was paid to the simultaneous presence of bacteria other than atypical *Pseudomonas* which could either contribute to the observed unspecific damage or even be the only responsible for disease development. *P. syringae* pv. *syringae* or *P. syringae* pv. *phaseolicola* were isolated from some of the bean's samples, *P. syringae* pv. *syringae* from some of the affected kiwifruits and *Erwinia* from some of the lettuces, although always in association with atypical *Pseudomonas*. In addition, the latter was the only bacteria collected from 28, 65, and 67% of bean, kiwifruit, and lettuce samples under investigation.

Genotypic identification of the *Pseudomonas* isolates. Nearly complete 16S rDNAs from the control strains *P. syringae* CECT 4429 and *P. viridiflava* CECT 458 and from six isolates with the atypical LOPAT pattern (Table 1), previously tested for pathogenicity, were sequenced after amplification. Analysis of the 16S rDNA sequences revealed that, independent of their origin, those from the atypical isolates were nearly identical (more than 99.9% identity over 1,439 nucleotides), and that they were most closely related to 16S rDNA sequences from several strains of *P. viridiflava* (including LMG2352 and CECT 458). Moreover, the sequence corresponding to hypervariable (hv) region 2, proposed by Moore et al. (14) as a signature for *P. viridiflava*, was found in the 16S rRNA genes of the new isolates. Also represented were the *P. viridiflava* sequences in hv regions 1 and 3, which distinguish this species from *P. syringae* or from *P. fluorescens* and some, but not all, of the *P. syringae* pathovars, respectively (14). In fact, with respect to *P. syringae*, higher identity values were observed with 16S rDNAs from strains of the *P. actinidiae*, *P. maculicola*, *P. morsprunorum*, and *P. theae* pathovars (more than 98% identity), all sharing hv region 3 with *P. viridiflava*. Finally, clustering of the atypical isolates 16S rDNA sequences with two control *P. viridiflava* sequences is revealed by the phylogenetic tree depicted in Fig. 2.

Comparisons of the sequences generated in this work with those of relevant *Pseudomonas* strains obtained from databases led to the identification of an additional hv region within the 16S rDNA of *P. viridiflava* strains, including the atypical isolates described in this work. It is located within the V5 region, helix 27 (corresponding to *Escherichia coli* 16S rRNA positions 829 to 857), described by Neefs et al. (15). This region contains a *Hin*fI site which is absent from the rDNA of *P. syringae* pathovars, including those of *P. actinidiae*, *P. maculicola*, *P. morsprunorum*, and *P. theae*, as well as *P. syringae* and *P. phaseolicola*. Moreover, the equivalent region from these pathovars contains a single *Sac*I site, which is absent from *P.*

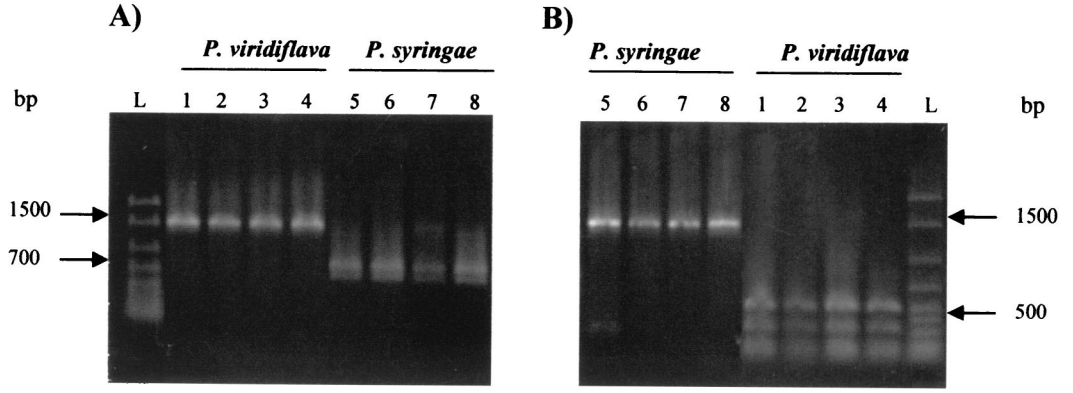

FIG. 3. Restriction profiles of PCR-amplified 16S rDNA fragments from different phytopathogenic *Pseudomonas.* (A) Digestion with *Sac*I; (B) digestion with *Hin*fI. Lane L, molecular size standards; lanes 1 to 3, RFLP profiles corresponding to atypical isolates of *P. viridiflava*; lane 4, *P. viridiflava* CECT 458; lane 5, *P. syringae* pv. *syringae* LPPA 93/01; lane 6, *P. syringae* pv. *syringae* LPPA 136/00; lane 7, *P. syringae* pv. *syringae* CECT 4429; lane 8, *P. syringae* pv. *phaseolicola* CECT 321.

viridiflava. A differential *Hin*fI site is also present in hv region 3 of *P. viridiflava* strains as well as in the *P. syringae* pathovars most closely related to *P. viridiflava* (see above). However it is absent from *P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola*. Such differential restriction sites were used to develop an RFLP ribotyping method that allows a rapid identification of the atypical isolates when combined with phenotypical tests. In fact, *Sac*I and *Hin*fI digestions of 16S rRNA genes amplified with pA and pH′ from all bacteria depicted in Table 1 confirmed this approach. Examples of *Sac*I and *Hin*fI digestions of 16S rDNAs from relevant strains are shown in Fig. 3.

Finally, in order to gain some insight into a possible genetic heterogeneity of the pathogenic *P. viridiflava* atypical isolates, RAPD typing with primer S was performed. In this way *P. viridiflava* organisms could be discriminated into at least six profiles (R-1 to R-6). Most of the strains, including the control *P. viridiflava* CECT 548, belong to R-1 (Fig. 4). In contrast, each of the *P. syringae* control strains generated a distinctive RAPD profile. The distribution of all bacteria under study into RAPD profiles is compiled in Table 1.

DISCUSSION

P. viridiflava strains are usually differentiated from *P. syringae* by their pathogenicity and by two of the biochemical tests represented in the LOPAT scheme. Whereas the first organism is considered as an epiphytic or opportunistic pathogen, the second, apart from being epiphytic, includes true pathogenic pathovars that cause different types of damage (such as necrosis, blights, rots, and spots) in a variety of plants. With respect to the LOPAT tests, *P. viridiflava* strains are L negative (i.e., they produce flat colonies on hypersucrose medium) and P positive (showing strong pectinolytic activity on potato slices). In contrast, *P. syringae* pathovars are L positive (forming convex colonies developing a strong white mucoid material on levan medium) and P negative. The emergent type described here shows a distinctive activity in both tests, with colonies characterized by a yellowish, rarely greenish, mucoid material in the L test, and a variable pectinolytic activity, ranging from negative or very weak to relatively strong (although it is generally weaker than that of the control *P. viridiflava* CECT 458 strain). It should be noted that the nature of the exopolysaccharide released by the atypical bacteria remains unknown, although its production in hypersucrose medium, but not in the same medium lacking sucrose, strongly suggests that it could be levan.

The species identification of the atypical phytopathogenic *Pseudomonas* isolates was achieved by analysis of the nucleotide sequences of 16S rRNA genes from representative isolates. Moore et al. (14) have previously demonstrated the potential of 16S rRNA sequence analyses to distinguish the species of the genus *Pseudomonas*, as well as for the establishment of phylogenetic lineages within the genus. In addition, sequence comparisons led to the identification of hypervariable regions that can be regarded as signatures for a certain species. In this work, similar experiments revealed the affiliation of the atypical isolates to *P. viridiflava* (Fig. 2). Accordingly, all signatures allowing discrimination of *P. viridiflava*

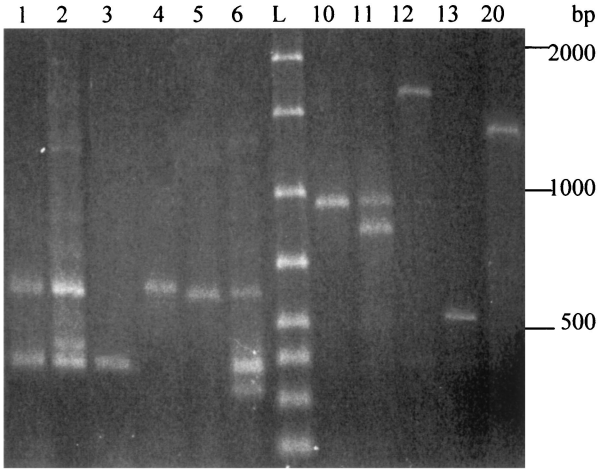

FIG. 4. RAPD typing of atypical *Pseudomonas* isolates performed with primer S. Lanes 1 to 6, profiles R-1 to R-6, respectively, generated by atypical *P. viridiflava* isolates; lanes 10 to 13, R profiles corresponding to *P. syringae* pv. *syringae* strains; lane 20, R profile of *P. syringae* pv. *phaseolicola*; lane L, molecular size standards. The distribution of the isolates within different profiles is shown in Table 1.

from closely related species (mainly the *P. syringae* pathovars and the *P. fluorescens* lineage [14, 17]) were found in the 16S rRNA genes from the new isolates. On the other hand, RAPD typing proved to be a useful tool to differentiate *P. syringae* from *P. viridiflava*, and also for differentiation within *P. viridiflava*.

To the best of our knowledge, the new type of *P. viridiflava* reported in this investigation has not been previously described as a phytopathogen. However, remarkable damage on kiwifruit during the flowering phase caused by typical *P. viridiflava* isolates has been reported (13, 24). According to Young et al. (24) the population of the pathogen surviving epiphytically on the plant could represent an important source of inoculum and may cause damage under particular environmental conditions. Thus, mild temperatures, frequent rainfalls, and high relative humidity values would facilitate their spread. These weather conditions are frequently found in the PA, where the atypical *P. viridiflava* is causing severe damage, with the accompanying economical loss. With respect to the origin of this lineage, one can speculate that an L negative *P. viridiflava* strain could have recently gained the ability to produce exopolysaccharide on hypersucrose medium, which may contribute to the epiphytic fitness of the strains or even function as a virulence factor (19, 4). Alternatively, erroneous laboratory interpretations of the L test, which could have been considered to be positive based only on the appearance of convex mucous colonies, but ignoring pigmentation, cannot be ruled out. If so, pathogenic strains related to *P. viridiflava* could have been claimed to be *P. syringae*. In fact, this possibility has been already indicated by Lelliott (11). The RFLP method developed in the present investigation, using 16S rDNA digestions with selected endonucleases (*Sac*I and *Hin*fI) might constitute a rapid and accurate genetic procedure to aid phenotypical characterization in the identification of the atypical isolates.

ACKNOWLEDGMENT

This research was supported by project INIA-SC00-026 of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Madrid, Spain).

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **de Andrés, M. F., F. García-Arenal, M. M. López, and P. Melgarejo.** 2000. Manual de laboratorio, p. 526. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.
3. **Deener, H. G., and I. Boychuck.** 1991. Species-specific detection of *Listeria monocytogenes* by DNA amplification. Appl. Environ. Microbiol. **57:**606–609.
4. **Denny, T. P.** 1995. Involvement of bacterial polysaccharides in plant pathogenesis. Annu. Rev. Phytopathol. **33:**173–197.
5. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853.
6. **Gardan, L., H. Shafik, S. Belouin, R. Broch, F. Grimont, and P. A. D. Grimont.** 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (*ex* Sutic and Dowson 1959). Int. J. Syst. Bacteriol. **49:**469–478.
7. **González, A. J., E. Landeras, and M. C. Mendoza.** 2000. Pathovars of *Pseudomonas syringae* causing bacterial brown spot and halo blight in *Phaseolus vulgaris* L. are distinguishable by ribotyping. Appl. Environ. Microbiol. **66**:850–854.
8. **Hildebrand, D. C., H. N. Schroth, and D. C. Sands.** 1988. Pseudomonas, p. 60–80. *In* N. W. Schaad (ed.), Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. American Phytopathological Society, St. Paul, Minn.
9. **King, E. D., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. Lab. Clin. Med. **44:**301–307.
10. **Lelliott, R. A., E. Billing, and A. C. Hayward.** 1966. A determinative scheme for fluorescent plant pathogenic bacteria. J. Appl. Bacteriol. **29:**470–478.
11. **Lelliott, R. A.** 1992. *Pseudomonas viridiflava*, p. 190. *In* I. M. Smith, J. Dunez, R. A. Lelliott, D. H. Phillips, and S. A. Archer (ed.), Manual de enfermedades de las plantas. Mundi-Prensa, Madrid, Spain.
12. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:**173–174.
13. **Mansilla, J. P., and A. Abelleira.** 1999. Presencia de *Pseudomonas marginalis* y *Pseudomonas viridiflava* sobre kiwi en Galicia. Bol. San. Veg. Plagas **25:** 175–180.
14. **Moore, E. R. B., M. Mau, A. Arnscheidt, E. C. Böttger, R. A. Hutson, M. D. Collins, Y. Van de Peer, R. de Wachter, and K. N. Timmis.** 1996. The determination and comparison of the 16s rRNA gene sequences of species of the genus Pseudomonas (sensu stricto) and estimation of the natural intrageneric relationships. Syst. Appl. Microbiol. **19:**478–492.
15. **Neefs, J.-M., Y. Van de Peer, L. Hendriks, and R. de Wachter.** 1990. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. **18:**2237–2330.
16. **Noval, C.** 1991. Manual de laboratorio, p. 379–410. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.
17. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1894, 237, p. 141–199. *In* N. J. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore, Md.
18. **Pecknold, P. C., and R. G. Grogan.** 1973. Deoxyribonucleic acid homology groups among phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol. **23:**111–121.
19. **Rudolf, K., M. Gross, F. Ebrahim-Nesbat, M. Noellenburg, and A. Zomorodian.** 1994. The role of extracellular polysaccharides as virulence factors for phytopathogenic pseudomonads and xanthomonads, p. 357–378. *In* C. I. Kado and J. H. Corsa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Press, Dordrecht, The Netherlands.
20. **Sawada, H., S. Kanaya, M. Tsuda, F. Suzuki, K. Azegami, and N. Saitou.** 2002. A phylogenetic study of the OCTase genes in *Pseudomonas syringae* pathovars: the horizontal transfer of the argK-tox cluster and the evolutionary history of OTCase genes on their genomes. J. Mol. Evol. **54:**437–457.
21. **Schaad, N. W., A. K. Vidaver, G. H. Lacy, K. Rudolph, and J. B. Jones.** 2000. Evaluation of proposed amended names of several pseudomonads and xanthomonads and recommendations. Phytopathology **90:**208–213.
22. **Soto, S. M., B. Guerra, M. A. González-Hevia, and M. C. Mendoza.** 1999. Potential of a three-way randomly amplified polymorphic DNA analysis as a typing method within twelve *Salmonella* serotypes. Appl. Environ. Microbiol. **65:**4830–4836.
23. **Williams, J. G. K., A. R. Kubelin, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.
24. **Young, J. M., G. J. Cheesmur, F. V. Welham, and W. R. Henshall.** 1988. Bacterial blight of kiwifruit. Ann. Appl. Biol. **112:**91–105.
25. **Young, J. M., Y. Takikawa, L. Gardan, and D. E. Stead.** 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Annu. Rev. Phytopathol. **30:**67–105.