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Characterization of *Erwinia chrysanthemi* by Pectinolytic Isozyme Polymorphism and Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments of *pel* Genes

ATEF NASSAR,[1,2] ARMELLE DARRASSE,[2]† MONIQUE LEMATTRE,[1] ALAIN KOTOUJANSKY,[2] CATHERINE DERVIN,[3] REGINE VEDEL,[2] AND YVES BERTHEAU[2]*

Station de Pathologie Végétale, Institut National de la Recherche Agronomique, 78026 Versailles Cedex,[1] *and Laboratoire de Pathologie Végétale*[2] *and Laboratoire de Biométrie,*[3] *Institut National de la Recherche Agronomique and Institut National Agronomique Paris-Grignon, 75231 Paris Cedex 05, France*

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Conserved regions about 420 bp long of the *pelADE* cluster specific to *Erwinia chrysanthemi* were amplified by PCR and used to differentiate 78 strains of *E. chrysanthemi* that were obtained from different hosts and geographical areas. No PCR products were obtained from DNA samples extracted from other pectinolytic and nonpectinolytic species and genera. The *pel* fragments amplified from the *E. chrysanthemi* strains studied were compared by performing a restriction fragment length polymorphism (RFLP) analysis. On the basis of similarity coefficients derived from the RFLP analysis, the strains were separated into 16 PCR RFLP patterns grouped in six clusters. These clusters appeared to be correlated with other infraspecific levels of *E. chrysanthemi* classification, such as pathovar and biovar, and occasionally with geographical origin. Moreover, the clusters correlated well with the polymorphism of pectate lyase and pectin methylesterase isoenzymes. While the pectin methylesterase profiles correlated with host monocot-dicot classification, the pectate lyase polymorphism might reflect the cell wall microdomains of the plants belonging to these classes.

Soft rot and wilt diseases caused by *Erwinia chrysanthemi* (Burkholder, McFadden, and Dimock) are widely distributed in many temperate and tropical areas (12, 28) and occur on a large number of hosts, including ornamental plants (19, 27, 30) and food crops (41, 44).

Previous attempts to group *E. chrysanthemi* strains on the basis of host range (13, 16) or on the basis of physiological (15), biochemical (39), and serological properties (40, 50) have shown that there is a great deal of variability in this species. The results of DNA-DNA liquid hybridization (7) and restriction fragment length polymorphism (RFLP) analyses of randomly chosen probes or rRNAs (6, 31) have also shown that this taxon is very diverse; the RFLP analyses grouped strains into clusters correlated with pathovar subdivisions (15, 16, 31).

However, these molecular tools which have been used previously can be used only with purified bacteria. Thus, a tool to specifically identify *E. chrysanthemi* that takes into account the previously reported diversity is still needed.

Several studies have shown that the pectinases, particularly the pectate lyases (PL), are involved in the phytopathogenicity of *E. chrysanthemi* (2, 5, 25). Electrofocusing of PL and RFLP analyses of PL-encoding genes (*pel*) both revealed polymorphism that might be used to differentiate *E. chrysanthemi* strains (6, 36). Furthermore, PCR-amplified fragments of *pel* genes were used recently to differentiate *Erwinia carotovora* subspecies in an RFLP analysis of the amplified fragments (10). Pathogenicity genes have also been used to differentiate other phytopathogenic bacteria (14).

The aim of this study was to investigate the ability of a *pel* gene PCR-based test to (i) identify *E. chrysanthemi* and (ii) differentiate the strains at the infraspecific level on the basis of the results of an RFLP analysis of the amplified fragments.

(Part of this work is based on a pending patent [9].)

MATERIALS AND METHODS

Bacterial strains. The pectinolytic *E. chrysanthemi* strains used in this study were studied previously (31) and are described in Table 1. Other *Erwinia* species and subspecies, including *E. carotovora* subsp. *atroseptica* (20 strains), *E. carotovora* subsp. *betavascularum* (2 strains), *E. carotovora* subsp. *carotovora* (10 strains), *Erwinia herbicola* (2 strains), and *Erwinia rhapontici* (1 strain), also were tested. Other pectinolytic bacteria that were associated or were not associated with soft rot symptoms, such as *Bacillus subtilis* (one strain), *Pseudomonas marginalis* (three strains), *Pseudomonas viridiflava* (one strain), *Pseudomonas fluorescens* (one strain), and *Yersinia ruckeri* (one strain), also were included. Nonpectinolytic microorganisms, including *Pseudomonas solanacearum* (three strains), *Xanthomonas campestris* (one strain), *Clavibacter* sp. (three strains), *Agrobacterium tumefaciens* (one strain), nitrogen-fixing bacteria (six strains), fungi and yeasts (five strains), *Pseudomonas* sp. (two strains), *Comamonas* sp. (two strains), and *Enterobacter* sp. (one strain), also were tested; the *Pseudomonas* sp., *Comamonas* sp., and *Enterobacter* sp. strains were kindly provided by J. M. Van der Wolf (IPO, Wageningen, The Netherlands) as strains that cross-reacted with antibodies raised against *E. chrysanthemi* (47, 50).

Chromosomal DNA isolation. Bacteria were grown overnight at 30°C in Luria broth (38). The cells in 2 ml of culture were collected by centrifugation, and the DNA was extracted as described previously (31), with the volume reduced by one-tenth. The quantity of DNA was determined by spectrophotometry, and the quality of the DNA was verified by electrophoresis.

DNA sequence analyses. The following previously published sequences were used: *pelA* (17), *pelA* and *pelE* (45), *pelE* (23, 35), and *pelADE* (49). These sequences were compared with the other *pel* gene sequences available in the GenBank release 75.0 and EMBL release 33.0 databases (*Ecapali*, *Ecapalx*, *Ecapela*, *Ecapelb*, *Echpel*, *Echpelb*, *Echpelc*, etc.).

Primer sequences in the open reading frames (ORFs) of *pel* genes were chosen after their amino acids were aligned by using programs developed by the University of Wisconsin Genetics Computer Group (GCG package) (11). The primer sequences were compared with the available sequences by using the BLASTN program (1) of the BLAST network service at the National Center for

* Corresponding author. Mailing address: Laboratoire de Pathologie Végétale, Institut National de la Recherche Agronomique and Institut National Agronomique Paris-Grignon, 16 Rue Claude Bernard, 75231 Paris Cedex 05, France. Fax: 33 (1) 44.08.17.00. Electronic mail address: bertheau@inapv.inapg.inra.fr.

† Present address: Station de Pathologie Végétale, de Phytoécologie et de Malherbologie, INRA, F97185 Point-à-Pitre Cedex, France.

TABLE 1. Strains of *E. chrysanthemi* used in this study

Host	Strain(s)[a]	Geographic origin and year	Source[b]	Biovar[c]	PL profile	PME profile	PCR RFLP pattern
Banana[d]	1871	Ivory Coast, 1976	1	3	15	3	15
Carnation	1240 (= ICPB EC 174)	Denmark, 1956	1	1	5	4	5
	1243 (= NCPPB 518)	Denmark, 1957	1	ND[e]	5	4	5
	1985	France, 1972	1	1	5	4	5
	2021	France, 1972	1	1	5	4	5
	795	France, 1965	1	1	5	4	5
	E II34	France, 1972	2	1	5	4	5
	1151	Italy, 1967	1	1	5	4	5
	30119, 30120, 30121, 30122	Italy	3	3	2	5	1
	1200 (= NCPPB 453)	United Kingdom, 1956	1	1	5	4	5
	PD 863 (= NCPPB 393)	United Kingdom, 1956	4	1	5	4	5
	1441	United States	1	5	16	4	16
Chicory	3262	France, 1981	2	5	18	4	16
	SF18-538	Switzerland	2	7	5	4	5
Chrysanthemum[d]	1346 (= ICPB EC 239)	Italy	1	5	18	4	16
	2048 (= NCPPB 402)	United States, 1956	1	5	18	4	16
	1242 (= NCPPB 427)	United States	1	5	18	4	16
Corn	1522	Columbia, 1973	1	3	13	3	13
	1271 (= NCPPB 1065)	Egypt, 1961	1	3	9	3	9
	1596	France, 1974	1	8	11	3	10
	1499	France, 1973	1	3	10	3	11
	1528 (= NCPPB 2541)	United States, 1966	1	8	11	3	10
	1268 (= NCPPB 1851)	United States, 1966	1	3	13	3	13
	2052 (= NCPPB 2538)	United States, 1970	1	3	12	3	14
	1534	Zimbabwe, 1955	1	3	10	2	11
	2595	Kenya	1	3	14	2	12
Dahlia	3367	France, 1977	3	1	5	4	5
	2013	France, 1974	1	1	5	4	5
Dieffenbachia	30608	France, 1974	3	3	8	5	7
	3642	France, 1974	3	3	8	5	7
	3665	France, 1974	3	2	7	5	8
	2014	France, 1974	1	3	8	5	7
	1237 (= NCPPB 1514)	Germany, 1962	1	2	7	5	8
	1152	Italy, 1968	1	2	7	5	8
	1870	Ivory Coast, 1976	1	2	7	5	8
	ED1	Martinique, 1987	5	3	6	5	6
	2051 (= NCPPB 2976)	United States, 1957	1	2	7	5	8
Kalanchoe	1805	Denmark, 1977	1	9	5	4	5
	3716	France, 1978	3	1	5	4	5
	30728, 30732, 30736	France, 1977	3	9	5	4	5
	30739	France	3	9	5	4	5
	2982	France, 1987	1	9	5	4	5
	2598	Switzerland, 1982	1	9	5	4	5
Parthenium	1270 (= NCPPB 516)	Denmark, 1957	1	6	17	5	16
	1236 (= NCPPB 1861)	United States, 1945	1	6	17	5	16
Pelargonium	1269 (= NCPPB 898 = B374)	Comoro Islands, 1960	1	3	4	5	3
Philodendron	EP2	Martinique, 1987	5	3	6	5	6
	PD 471	The Netherlands	2	3	6	5	6
	1248 (= NCPPB 454)	United States, 1957	1	3	6	5	6
	1245 (= NCPPB 204)	United States, 1959	1	3	6	5	6
Potato	2267 (= NCPPB 3346)	Australia, 1978	1	3	3	3	11
	2711 (= DAR 305-14)	Australia	1	3	3	3	11
	1888 (= NCPPB 3344)	France, 1978	1	1	5	4	5
	2288	France, 1980	1	1	5	4	5
	2015 (= NCPPB 3345)	France, 1975	1	7	5	4	5
	2593 (= CIP 004)	Peru	1	3	4	5	3
	2594	Peru	1	3	9	3	9
	CIP 366, CIP 367	Peru	6	3	3	5	4

Continued on following page

TABLE 1—*Continued*

Host	Strain(s)[a]	Geographic origin and year	Source[b]	Biovar[c]	PL profile	PME profile	PCR RFLP pattern
Saintpaulia	30909, 30913	France, 1974	3	3	1	5	2
	3937	France, 1977	3	3	1	5	2
	30932	France, 1983	3	3	1	5	2
Sunflower	SF109-1	France, 1986	2	ND	19	4	16
Tobacco	1891	United States	1	3	6	5	6
Tomato	722	France, 1965	1	1	5	4	5
	SA86-10	France, 1981	2	5	18	4	16
	ET1, ET2	Martinique, 1987	5	3	3	5	3
	ET3, ET5	Martinique, 1987	5	3	6	5	6
	ET11	Martinique, 1988	5	3	3	5	3
	SH230-C143	Cuba	2	2	2	5	1

[a] Abbreviations: ICPB, International Collection of Phytopathogenic Bacteria, University of California, Davis; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; CIP, Centro Internacional de la Papa, Lima, Peru.

[b] 1, Collection Française de Bacteries Phytopathogènes, Institut National de la Recherche Agronomique (INRA), Angers, France; 2, R. Samson, INRA, Angers, France; 3, M. Lemattre, INRA, Versailles, France; 4, Culture Collection Plant Pathogenic Service, Wageningen, The Netherlands; 5, IRAT-CIRAD, Martinique, France; 6, B. Jouan, INRA, Rennes, France.

[c] As described by Ngwira (33) and Boccara et al. (6).

[d] Strains isolated from banana in Colombia (strains 1445 and 1451; Collection Française de Bacteries Phytopathogènes, INRA, Angers, France) and Guadeloupe (strain EM1; Laboratory Collection, INRA, Guadeloupe, French West Indies) and from chrysanthemum in France (strains 40245 and 40251; Laboratory Collection, INRA, Versailles, France) and initially provided as *E. chrysanthemi* strains were not amplified by our test. These strains were later reported to differ from other *E. chrysanthemi* strains in their genetic and phenotypic characteristics (31, 26), and were no longer considered members of this species.

[e] ND, not determined.

Biotechnology Information, Bethesda, Md., and the FASTA program of the European Molecular Biology Organization network service.

PCR amplification of DNA. The PCR medium used was the medium recommended for *Taq* polymerase by Cetus Corp., Perkin-Elmer, Norwalk, Conn. Each reaction was performed by using a 100-μl reaction mixture overlaid with 50 μl of mineral oil (Sigma, St. Louis, Mo.). The samples contained 100 ng of target genomic DNA, 200 μM deoxynucleotide triphosphates, and 100 pmol of each primer. After the samples were boiled for 3 min, 2.5 U of *Taq* polymerase (Perkin-Elmer or Boehringer, Mannheim, Germany) was added to each preparation. The resulting mixture was subjected to 25 cycles consisting of incubations for 1 min at 94°C and incubation for 2 min at 72°C in a Gene ATAQ controller [Pharmacia LKB] or a Hybaid thermal reactor (Céra Labo, Ecqueirlly, France). The last stage included both PCR primer annealing and elongation steps. The initial analysis of PCR products was performed by electrophoresing 5-μl portions of amplification medium in 1% (wt/vol) agarose minigels (29).

Restriction fragment analysis. A 95-μl portion of PCR-amplified DNA was precipitated with ethanol and resuspended in 15 μl of TE buffer (29). Then the DNA was digested with *Alu*I, *Eco*RV, *Hpa*II, *Sau*3AI, or *Taq*I for 2 to 3 h in a 20-μl mixture containing 5 μl of the DNA solution, as recommended by the supplier (Boehringer Mannheim). Portions (12 μl) of each sample were electrophoresed on 9% polyacrylamide gels by using 1 μg of marker V (Boehringer Mannheim) as a size marker. All gels were stained with ethidium bromide as described previously (10).

Phenetic and phylogenic data analyses. Each restriction fragment was treated as a unit character and was given, for all strains, a score of 1 if it was present and a score of 0 if it was absent (42). The resulting data matrices were analyzed by different phenetic and cladistic methods.

(i) Phenetic analyses. A pairwise distance (D) matrix ($D = 1 - S$) was obtained by using Statistical Analysis Software version 6 (SAS Institute, Inc., Cary, N.C.), the 0/1 matrix, and the Sorensen-Dice similarity coefficient (S) (21). The resulting distance matrices were used as input data for hierarchical clustering by the unweighted average pair group method. Trees were drawn with the help of the Macro GRFTREE developed by D. Jacobs for SAS Institute, Inc.

A factorial correspondence analysis was performed with the 0/1 matrix by using the SAS package. The phena were distributed by projecting each strain on the first two or three more explanatory axes.

(ii) Cladistic analyses. Raw data were analyzed by the Wagner parsimony portion of the "Mix" program available in the PHYLIP package, version 3.51C (18). Ten runs were done for the whole set of data with different strain input orders ($J = 10$), and the consensus tree was chosen by using the "Consense" program. The resulting tree was drawn by using the "Drawtree" program.

Detection of isozyme polymorphism. The PL and pectin methylesterase (PME) isozymes were analyzed by using crude or concentrated samples obtained from the supernatant or from cell lysates (samples were concentrated up to 100-fold against polyethylene glycol). Cell lysates and supernatants were obtained from early-stationary-phase cultures grown in pectinase-inducing medium (4). Portions (15 μl) of samples were layered on an ultrathin (thickness, 0.5 mm) polyacrylamide gel and electrofocused with a pH gradient of 3 to 10 or 8 to 10.5 at a constant power of 30 W for 1 h (nonequilibrated conditions). The PL and PME activities were assessed directly on the gel as described previously (4).

RESULTS AND DISCUSSION

PCR amplification. The ORFs of the six available sequences (20) of the *E. chrysanthemi pelADE* gene cluster were aligned pairwise. The primer sequences used were chosen because they were in the conserved regions of these ORFs. The primers were also chosen because the distance between them was short enough for easy amplification but not so short as to restrict polymorphism analysis in the target region. The following two oligonucleotides (a 23-mer and a 30-mer), which were sepa-

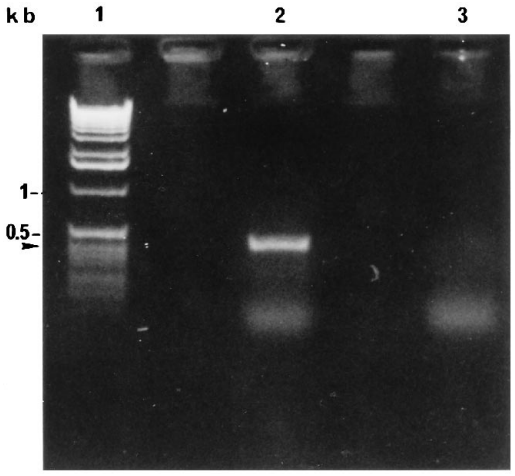

FIG. 1. PCR amplification of *pelADE* fragments with primers ADE1 and ADE2. The PCR products (after 25 cycles) were separated by electrophoresis on a 1% agarose gel. Lane 1, 1-kb DNA ladder; lane 2, *E. chrysanthemi* 3937; lane 3, *Escherichia coli* K-12. The arrowhead indicates the position of the 420-bp amplified fragment.

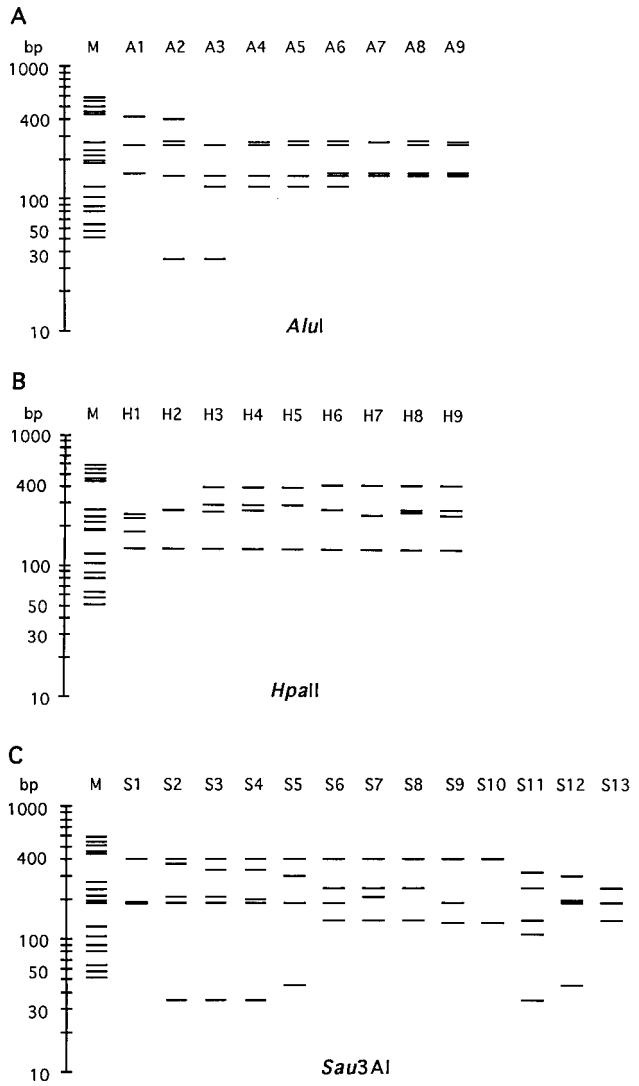

FIG. 2. RFLP analyses of *pelADE* amplified fragments. DNA products were digested with restriction enzymes *Alu*I (A), *Hpa*II (B), and *Sau*3AI (C) and separated on a 9% polyacrylamide gel. Lane M, molecular weight marker V (Boehringer Mannheim); lanes A1 through A9, *Alu*I PCR RFLP patterns 1 through 9, respectively; lanes H1 through H9, *Hpa*II PCR RFLP patterns 1 through 9, respectively; lanes S1 through S13, *Sau*3AI PCR RFLP patterns 1 through 13, respectively. See Table 2.

rated by 420 bp, were selected as PCR primers: ADE1 (5′-GATCAGAAAGCCCGCAGCCAGAT-3′) and ADE2 (5′-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3′). The sequence of each primer was checked for identity with the sequences in the complete GenBank release 75.0 and EMBL release 33.0 databases, allowing up to five mismatches. No highly homologous sequence, particularly at the 3′ end, was found in these databases except for the *pel* sequences used in this study.

The specificity of the primers was assessed by amplifying the DNAs of the following two bacterial strains under different temperature and duration conditions: *E. chrysanthemi* 3937 and *Escherichia coli* K-12 (Fig. 1). The conditions described above were then retained. The specificity of the PCR test was then checked with the whole collection of microorganisms.

A ca. 420-bp amplified fragment was obtained for all 78 *E. chrysanthemi* strains tested. No amplified fragment was observed with the other organisms tested. Thus, our PCR test could be used to identify this species, as was found by other investigators (43, 48).

The design of the primers, in which conserved sequences of the ORFs of the *pelA*, *pelD*, and *pelE* genes were used, should allow amplification of all strains, including those with truncated genes (45). Moreover, as these genes have been shown to play a role in virulence (5), it is expected that the PCR test should reliably identify all classes of pathogenic *E. chrysanthemi* strains.

RFLP analysis. The *E. chrysanthemi* strains were analyzed by performing an RFLP analysis. The 420-bp amplified fragments were digested with several enzymes chosen on the basis of the restriction maps of the genes sequenced. *Eco*RV did not reveal any polymorphism when it was tested with a representative collection of strains. In contrast, *Taq*I gave many restriction fragments which were difficult to analyze; therefore, this enzyme was not used. Electrophoresis experiments revealed both strong and light restriction bands. To avoid misinterpretation, only the strong electrophoresis bands were recorded. *Alu*I, *Hpa*II, and *Sau*3AI each gave 9 to 13 different patterns (Fig. 2) for the whole collection of strains. The combined results obtained with these three restriction enzymes revealed that there were 16 RFLP patterns (Table 2).

The sums of the sizes of the restriction fragments suggest that the polymorphism observed for each strain was due to one, two, or three different amplified fragments. As the number of *pel* genes belonging to the *pelADE* family expressed by the bacteria differs in different strains (3, 36), the results show that the polymorphism observed might have resulted either from the presence of up to three *pel* genes that are more or less conserved in strains or from the presence of truncated genes

TABLE 2. Summary of the RFLP groups and phenotypic profiles of the 78 *E. chrysanthemi* strains studied

Cluster	PCR RFLP pattern[a]	No. of strains	PCR RFLP pattern for the following restriction enzymes[b]:			PL profile(s)[c]	PME profile(s)[c]
			*Alu*I	*Hpa*II	*Sau*3A		
1	1	5	5	4	4	2	5
	2	4	2	4	4	1	5
	3	5	6	5	3	4, 3	5
	4	2	6	5	2	3	5
2	5	24	3	2	1	5	4
3	6	8	7	5	12	6	5
	7	3	7	3	5	8	5
	8	5	4	3	5	7	5
4	9	2	9	8	6	9	3
	10	2	9	7	6	11	3
	11	4	9	9	6	3, 10	2, 3
	12	1	9	7	13	14	2
	13	2	9	7	9	13	3
	14	1	9	7	10	12	3
5	15	1	8	1	7	15	3
6	16	9	1	6	11	16, 17, 18, 19	4, 5

[a] The PCR RFLP patterns correspond to those indicated in Table 1 and Fig. 3.
[b] See Fig. 2.
[c] See Fig. 5.

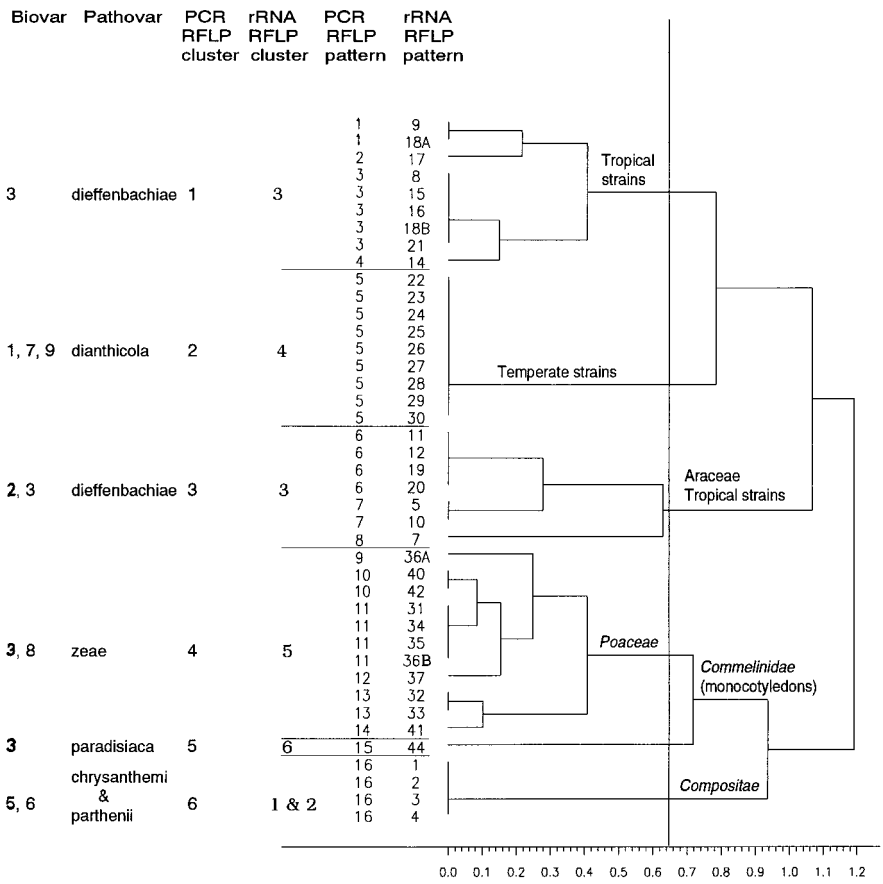

FIG. 3. Dendrogram derived from PCR-RFLP analyses of the 78 *E. chrysanthemi* strains studied by using the unweighted average pair group method and the Sorensen-Dice similarity coefficient. The scale indicates genetic distances. Also indicated are the biovar number, the PCR RFLP cluster, the rRNA RFLP cluster (31), the PCR RFLP pattern as indicated in Table 2, and the rRNA RFLP group (31).

(45). The sizes of the restricted fragments always added up to 420, 840, or 1,260 bp. The missing base pairs could have resulted from (i) superimposed bands at the same location and (ii) not counting low-molecular-weight bands that could not be distinguished from the primers; thus, a restriction map could not be constructed from the data obtained.

Phenetic and cladistic analyses. To facilitate comparison with our previous rRNA RFLP pattern results (31), the dendrogram in Fig. 3 includes the rRNA RFLP pattern numbers, as well as the rRNA RFLP cluster numbers. We compared the simple factorial correspondence analysis, Wagner parsimony, and genetic distances for the whole data set. Globally, the main clusters of our strains were conserved, and the trees obtained by the two methods used (phenetic and phylogenic analyses) appeared to be relatively reliable. The classification obtained by using the distance, $D = 1 - S$, where S is the Sorensen-Dice similarity coefficient, and the unweighted average pair group clustering method with the whole data set is shown in the dendogram in Fig. 3. Cluster definitions at a distance of 0.65 allowed us to separate the *E. chrysanthemi* strains into six clusters.

Except for the different locations of several leaves within individual clusters, the clustering of the strains based on PCR RFLP data was quite similar to the clustering based on rRNA RFLP pattern data (31). Thus, all of the previous remarks concerning the relationships between the clusters and the pathovars, biovars, and geographical origins also applied to this clustering.

The cluster 1 and 3 strains have similar PME 5 patterns and belong to biovars 3 and 2 (Table 2). The strains belonging to these two clusters previously were affiliated on the basis of their DNA-rRNA hybridization patterns with *E. chrysanthemi* pv. dieffenbachiae and with strains isolated from plants that originated from tropical areas (31). These two clusters, which were separated on the dendrogram in Fig. 3, were grouped by the cladistic analyses (Fig. 4) and the factorial correspondence analyses (data not shown).

Moreover, all of the strains isolated from *Dieffenbachia* and *Philodendron* spp., which belong to the same plant family, the Araceae, are grouped in cluster 3. Thus, this cluster encompasses the two closely related taxa *E. chrysanthemi* pv. philodendroni and *E. chrysanthemi* pv. dieffenbachiae (46). Tomato strains ET3 and ET5, which were isolated in Martinique (French West Indies) and belonged to this cluster, shared with the philodendron strains not only the same PCR RFLP pattern but also the same PL profile. These results are consistent with the findings of Boccara et al. (6). Thus, although more strains should be studied, our PCR RFLP technique could distinguish between *E. chrysanthemi* pv. philodendroni (PCR RFLP pattern 6) and true *E. chrysanthemi* pv. dieffenbachiae (PCR RFLP patterns 7 and 8).

Most (but not all) of the strains belonging to these clusters produced five PL isoenzymes. The high number of PL isoenzymes produced by these strains may allow them to invade a broad range of plants. This hypothesis is supported by the diversity of hosts from which the strains of these clusters were

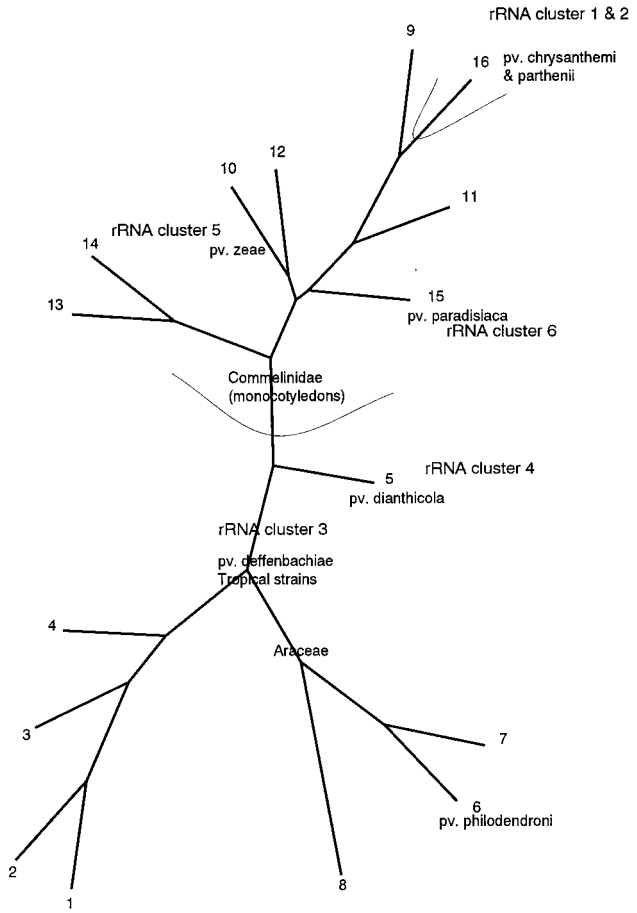

FIG. 4. Cladogram derived from RFLP analyses of the 78 *E. chrysanthemi* strains studied by using the Wagner parsimony method ("Mix," "Consense," and "Drawtree" programs of the PHYLIP package). The numbers are the PCR RFLP pattern numbers (Table 1 and Fig. 3).

isolated and by the results of pathogenicity tests carried out with a broad set of hosts (31).

The great diversity observed in the strains obtained from the tropical areas (clusters 1 and 3), particularly strains isolated from members of the Solanaceae (cluster 1), suggests that tropical regions might be a center of diversification of these *E. chrysanthemi* strains. A similar observation was made previously for *E. carotovora* strains (34). Although more strains need to be studied, we suggest that South America and/or Central America might be a center of diversification of these pectinolytic bacteria, as it is for several members of the Solanaceae.

Cluster 2, the largest and most homogeneous cluster, included 24 strains isolated from carnation, kalanchoe, dahlia, tomato, potato, and chicory plants. These strains, all of which were isolated in temperate regions, produced identical patterns. Strains belonging to this cluster were previously considered members of *E. chrysanthemi* pv. dianthicola (31) and belong to the "temperate" group of strains (22). The kalanchoe strains were not more distinguished in this study in contrast to the previous analysis based on their rRNA RFLP patterns (31).

The different strains of this cluster were able to induce wilting symptoms in carnation plants (31), a characteristic of *E. chrysanthemi* pv. dianthicola (12). All of these strains share common traits, such as PL 5 and PME 4 profiles, and are members of biovars 1, 7, and 9, which are closely related (31). All of these characteristics distinguished the strains of this cluster from all other *E. chrysanthemi* strains. These strains can also be differentiated by their growth requirements, by DNA hybridization similarity coefficients ranging from 90 to 100% (6), and by rRNA RFLP cluster data (31). Consequently, the uniformity of this cluster might appear to be enough to justify subspecies status for these strains.

All of the strains isolated from corn or previously identified as members of *E. chrysanthemi* pv. zeae (8) were grouped in cluster 4 and were isolated from members of the plant family Poaceae. These strains contained PME 3 and PME 2, belonged to two closely related biovars, biovars 3 and 8, and exhibited a high level of diversity in their PL profiles. A similar polymorphism was observed previously in their rRNA RFLP patterns (31). Corn also originated from South America and Central America, like the Solanaceae. The origin of polymorphism among strains should be investigated further, but these results support our hypothesis formulated above for the strains isolated from members of the Solanaceae that these regions might be a center of diversification of *E. chrysanthemi.*

Cluster 5 contained only one banana strain obtained from Ivory Coast. This strain has a unique PL isozyme profile and was reported previously to be clearly distinct (6). However, more strains of *E. chrysanthemi* isolated from banana must be studied to justify the conclusion that cluster 5 corresponds to *E. chrysanthemi* pv. paradisiaca, as previously shown (31). Cluster 5 is closely related to cluster 4, and these two clusters have the same PME profile (PME 3 and sometimes PME 2) and contain two closely related biovars, biovars 3 and 8 (32). Thus, clusters 4 and 5 may be grouped together as a taxon that consists of strains restricted to the plant subclass Commelinidae (monocotyledons).

Cluster 6, which was as homogeneous as PCR RFLP cluster 2, consisted of strains belonging to *E. chrysanthemi* pv. parthenii and *E. chrysanthemi* pv. chrysanthemi. All of the strains were isolated from plants belonging to the Compositae, one of the most highly evolved groups of dicotyledons.

The parthenium strains had the same PL 17 and PME 5 profiles and were classified in biovar 6, which distinguished them from the other strains in the same cluster. The same parthenium strains were included in a separate but closely related cluster in the previous rRNA RFLP analysis (31). The other strains belonging to this cluster were members of *E. chrysanthemi* pv. chrysanthemi (12), a quite homogeneous pathovar, as reported previously (31), which is characterized by PME 4. Thus, in contrast to our previous results (31), strains belonging to these two closely related pathovars cannot be distinguished by their PCR RFLP patterns but still can be differentiated by their PL and PME profiles.

Only the following two differences were observed between the rRNA RFLP clustering (31) and the PCR RFLP clustering (Fig. 3): (i) rRNA RFLP cluster 3 was divided into PCR RFLP clusters 1 and 3, and (ii) closely related rRNA RFLP clusters 1 and 2 (31) were grouped into PCR RFLP cluster 6 (Fig. 3). However, compared with the unweighted average pair group method dendrogram (Fig. 3), the cladistic cladogram (Fig. 4) and the factorial correspondence analysis results (data not shown) provided a more appropriate graphic representation of the relationship between PCR RFLP clusters 1 and 3. These two analysis methods revealed a wide but continuous distribution of the strains in these clusters, which were grouped in these graphic representations as they were grouped previously (31).

PL polymorphism. PL profiles are shown in Fig. 5. A great deal of polymorphism was observed, and 19 profiles were dis-

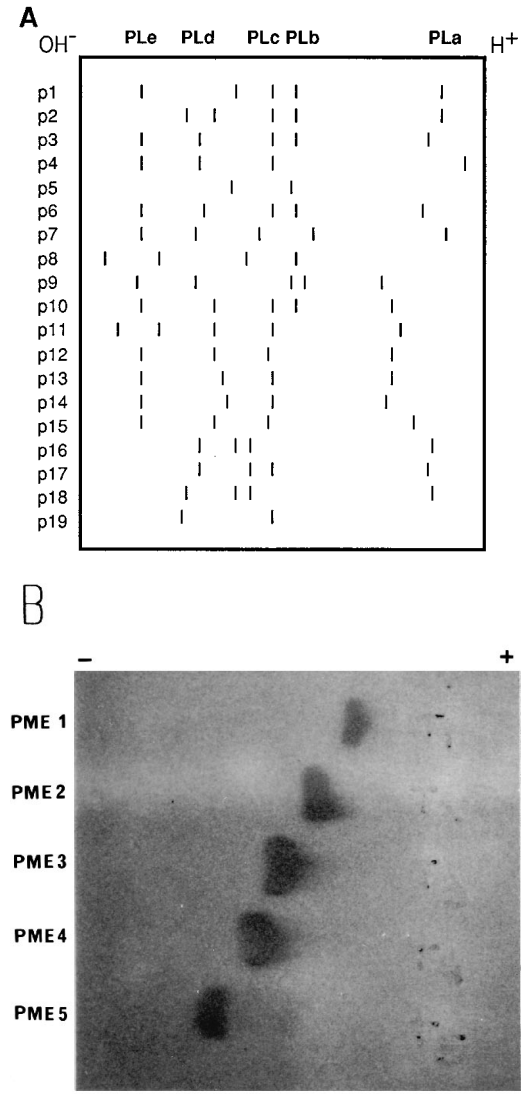

FIG. 5. Representative PL profiles (A) and PME profiles (B) of 78 *E. chrysanthemi* strains obtained by electrofocusing of supernatants. (A) The profile numbers (p) are indicated on the left (Table 2).

tinguished (Table 2). Reference strain 3937 (Fig. 5A, p1) produced the classical five PL isozymes (PLa, PLb, PLc, PLd, and PLe). Because of the slight variation in the apparent pI, determination of the PL profiles was highly fastidious, requiring electrofocusing of numerous samples on the same gels. Thus, in our opinion, PL profile analysis might not be of practical value for routine tests.

PME polymorphism. Four kinds of PME, designated PME 2, PME 3, PME 4, and PME 5 on the basis of their increasing pI values, were observed (Fig. 5B and Table 2). PME 1 was defined previously (6) for strain EM1, which does not belong to *E. chrysanthemi.*

Correlation of PCR RFLP clusters with other characteristics of the strains. The six PCR RFLP clusters shown in Fig. 3 correlated very well with the pathovars (12, 16), biovars (33, 40), and RFLP groups obtained previously by using a larger DNA region (16+23S rRNA) (31).

The PL profiles also corresponded quite well to the PCR RFLP patterns (Table 2 and Fig. 3). However, PL polymorphism within PCR RFLP patterns 3, 11, and 16 was observed, and this polymorphism could be correlated with the original hosts (Table 1). The PME profiles correlated with the monocot-dicot origins of the plants (Tables 1 and 2 and Fig. 3). Our analyses of these relationships are summarized above.

In conclusion, our PCR test reliably identified *E. chrysanthemi*, while the polymorphism of the amplified fragment clearly distinguished the pathovars. Methods for identifying pathogenic bacteria have been developed previously by using genes involved in pathogenicity (10, 14). However, as these genes are not neutrally selected, their use might introduce a bias in taxonomic studies that was not observed in our study. This is the first demonstration that neutral markers (rRNA) (31) and selected markers (the *pel* genes involved in phytopathogenicity) (5) result in the same classification of *E. chrysanthemi* strains. Such similar results obtained with independent phenotypic and genotypic markers emphasizes the viability of the pathovar classification of this species that is still being debated (33).

The PCR RFLP clustering results correlated very well with the isoenzyme profile results. While the bacterial PME profiles clearly revealed differences between monocot isolates (PME 2 and PME 3) and dicot isolates (PME 4 and PME 5), the PL profiles revealed the diversity of the monocot and dicot isolates. A similar polymorphism of PL profiles has been reported previously (3, 6, 36), and this polymorphism might reflect the adaptation of the bacteria to the plant cell wall microdomains (37). Unfortunately, because of the very slight pI differences between PL, PL profiles are very fastidious to determine and might not be of practical use. Thus, in addition to differential regulation of the *pel* genes (3, 24), the PL polymorphism, which is correlated with the polymorphism of the genes, might largely explain the wide range of plants infected by *E. chrysanthemi* strains.

Finally, consistent with the rRNA RFLP patterns (31), the results revealed the significant polymorphism of strains isolated from plants (corn, potato, and tomato) whose center of diversification is located in Central America and South America. Although more strains, particularly more strains from these regions, need to be studied, such diversity might indicate coevolution and that these regions are centers of diversification of *E. chrysanthemi*.

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