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# Ribotyping of *Erwinia chrysanthemi* Strains in Relation to Their Pathogenic and Geographic Distribution

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**16S and 23S rRNAs from *Escherichia coli* were used to study the relationship among a representative collection of strains of *Erwinia chrysanthemi* differing in their original host and geographical origin. Phenetic analysis of restriction fragment length polymorphisms allowed the distribution of the studied strains into seven clusters. These clusters were similar to those obtained by cladistic methods and appeared to correlate well with the established pathovars and biovars but to a lesser extent with geographical distribution. Except for two groups of strains defined as tropical and temperate isolates (clusters 3 and 4, respectively), our clustering correlated well with botanical classifications of host plants. However, the rRNA groupings were shown to be more discriminative than biovar analysis. To assess the relationship between rRNA clusters and pathogenicity, 12 representative strains from different clusters were tested for pathogenicity on different plants. The two typical symptoms, maceration and wilting, were observed for these strains. The occurrence of the tobacco hypersensitivity reaction for a subset of these strains is discussed in light of recent results concerning the presence of an *hrp* gene. Considering symptom expression only, rather than the capacity for plant infection, strains from the same cluster were shown to induce similar symptoms in test plants. Thus, since host specificity is still quite controversial, rRNA patterns may constitute a useful tool in taxonomic and epidemiological studies of *Erwinia chrysanthemi* species.**

*Erwinia chrysanthemi*, originally isolated from chrysanthemum by Burkholder et al. (8), is a member of the pathogenic enterobacteria causing soft rot, stunting, and wilting on a wide range of plants in different parts of the world (4). The host specificity of this species is still under debate.

Dye (14) classified *E. chrysanthemi* as a variety of *Erwinia carotovora*. The status of *E. chrysanthemi* and *E. carotovora* as separate species is further supported by differences in DNA homology (7, 18, 42) as well as other approaches such as numerical analyses of biochemical features (47), two-dimensional electrophoresis of bacterial proteins (28), biochemical and physiological characteristics (30), pectic enzyme production (36), differences in pectolytic activity at variable temperatures (33), bacteriophage sensitivity (32), and fatty acid profiles (27).

Several attempts have been made to discriminate among strains of *E. chrysanthemi*, but the results have been inconsistent because these studies resulted in largely unrelated characteristics. *E. chrysanthemi* has been divided, on the bases of its pathogenicity on some host plants and limited biochemical and physiological differences, into six subdivisions or pathovars: pv. chrysanthemi, pv. zeae, pv. dieffenbachiae, pv. parthenii, pv. paradisiaca, and pv. dianthicola (15). However, this pathovar classification is still debated (30), and the relationships between pathogenicity and phenotypic properties of strains or pathovars are not entirely clear (12, 39, 48). An alternative biovar classification based on biochemical and physiological criteria independent of pathogenicity has been proposed, and nine biovars were distinguished (30, 39).

Similarly, no specific relationships were found between serological properties determined with antisera raised against whole cells (39), lipopolysaccharide (5), pectate lyase (PL) (25), or membrane protein complex (48) and host of origin or phenotypic characteristics (40). However, Uesugi et al. (44, 45), using a limited number of *E. chrysanthemi* strains, showed a possible correlation between the electrophoretic profiles of membrane protein and the hosts from which the strains were isolated. It was suggested that some of the membrane proteins are important determinants of host specificity.

Various molecular approaches have been used in the intraspecific differentiation of *E. chrysanthemi*. Brenner et al. (6) classified strains of *E. chrysanthemi* into four groups on the basis of DNA hybridization. These included strains from chrysanthemum and guayule, from dahlia and dieffenbachia, from corn and grasses, and a single strain from sugarcane. Strains of each group were highly interrelated but all were distinct from *E. carotovora*.

Human (21) and pathogenic plant (2, 35) bacteria have been successfully typed by using rRNA as a tool for restriction fragment length polymorphism (RFLP) analyses. Moreover, 2-acetylaminofluorene (AAF)-labelled rRNA from *Escherichia coli* (20) has already been successfully used to determine gene restriction patterns for the taxonomic study of *Xanthomonas campestris* pathovars (2). The objective of this study was to characterize strains of *E. chrysanthemi* from different plant species and geographical areas by using this approach to investigate the relationship, if any, between the genetic diversity of *E. chrysanthemi* and its hosts or geographical distribution.

## MATERIALS AND METHODS

**Bacterial strains.** Origins and references for the 82 strains of *E. chrysanthemi* used in this study are shown in Table 1. Twenty

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strains of *E. carotovora* subspecies and one atypical banana strain of *E. chrysanthemi* were also used (data not shown).

The strains were grown in Luria-Bertani broth (37) with shaking overnight at 30°C.

**Biovar determination.** Strains with an unknown biovar classification were characterized as described by Ngwira and Samson (30).

**DNA preparation.** Cells were harvested from 2 ml of an overnight culture by microcentrifugation for genomic DNA extraction by using a modification of the method of Klotz and Zimm (26). Briefly, the bacteria were resuspended and washed twice in 1 ml of 50× TE buffer (500 mM Tris-HCl, 50 mM EDTA [pH 8]), lysed for 15 min on ice after 100 μl of lysis solution (0.01% RNase, 0.1% lysozyme, 10% sucrose in 50× TE) and then sodium dodecyl sulfate to a final concentration of 1% and 50 μg of proteinase K were added, and incubated at 37°C until the preparation was clear. The mixture was deproteinized by sequential phenol and chloroform-isoamyl alcohol (24:1 [vol/vol]) extraction. The DNA was precipitated in ethanol, resuspended in TE buffer, and quantified by spectrophotometry at 260 nm.

**Gel electrophoresis of endonuclease-cleaved DNA.** Samples of DNA (approximately 3 μg) were digested by each of the three restriction endonucleases (5 to 10 U/μg of DNA) *Eco*RI, *Hin*dIII, and *Bam*HI (Boehringer) according to the manufacturer's instructions. Horizontal agarose gel electrophoresis of the DNA fragments was performed by using 0.8% (wt/vol) agarose (Appligène, Illkirch, France) in Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA [pH 8]). The molecular weight standard Raoul I (Appligène), containing 22 DNA fragments (234 to 48,502 bp), was included in the gels. The DNA digest was visualized by staining with ethidium bromide and exposing the gel to UV light.

**Probes.** AAF-labelled 16S and 23S rRNAs from *E. coli* (Eurogentec, Seraing, Belgium) were used to detect fragments in the genomic DNA of bacteria. AAF-labelled pBR322 DNA (Eurogentec) was hybridized to 21 DNA fragments of the standard Raoul I set to standardize Southern membranes.

**Southern transfer and hybridization.** The total DNA extracts, separated on the gels, were denatured and transferred to a BA83 nitrocellulose membrane (Schleicher & Schuell) as described by Southern (41). The transfer membranes were prehybridized and hybridized as described elsewhere (2).

**Immunodetection of rRNA-rDNA duplexes.** Hybridized bands were immunodetected as described previously (2). Briefly, the membranes were incubated for 1 h with purified anti-AAF mouse monoclonal antibody (Eurogentec), washed three times, and incubated for 1 h with alkaline phosphatase-labelled sheep antibodies raised against mouse immunoglobulin G (Biosys). After a further washing, membranes were incubated with nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The enzymatic reaction was stopped by washing the membranes in distilled water.

**DNA fragment size determination.** Sizes of the hybridized fragments from each strain were determined by using a LECPHOR 500 (Biocom, Les Ulis, France) integrating spectrophotometer equipped with a band-tracking scanner. DNA fragment size standard Raoul I was used to determine the interpolation function relating size to migration.

**Phenetic and cladistic analyses.** Each hybridizing band was treated as a unit character and scored as 1 (present) or 0 (absent) across all strains. The resulting matrix was then analyzed by phenetic and cladistic methods. The pair-wise distance matrices, using either the complement to one or Nei and Li (29) distances of the Jaccard, Ochiai, Sorensen-Dice, and Kulczinsky similarity coefficients (22), were calculated by using Statistical Analysis Software, version 6 (SAS Institute Inc., Cary, N.C.). The distance matrices were then used as data for hierarchical clustering, using Ward's minimum variance, unweighted average pair group method, single linkage, and complete linkage methods. The resulting trees were drawn using the Macro GRFTREE program developed by D. Jacobs for SAS. Furthermore, a simple factorial correspondence analysis was performed on the 0/1 matrix by using SAS. Distribution of the phenons was performed by projection of each strain on the three axes that explain most of the variance.

Finally, the data were analyzed by the Wagner parsimony method (MIX program) of the PHYLIP 3.5 software package (16). Twenty runs were done with different strain input orders, and the resulting trees were submitted to analysis with the CONSENSE program before drawing the tree with the DRAWTREE program (both of the PHYLIP 3.5 software package).

**Pathogenicity tests.** Twelve strains of *E. chrysanthemi* selected from different geographical origins, including (i) strains representative of the different RFLP patterns isolated from the same host plant and (ii) strains showing the same patterns or from the same cluster but isolated from different host plants, were assessed for pathogenicity and hypersensitivity (HR). Bacteria grown for 20 h at 30°C on Luria-Bertani agar plates were suspended in sterile physiological saline (PS) and adjusted to approximately $10^8$ CFU $ml^{-1}$. Pathogenicity and HR tests were performed on the following plant species: *Nicotiana tabacum* L. (tobacco; 8- to 10-leaf stage, cultivar Xanthi), *Dianthus caryophyllus* (unrooted carnation cuttings; cultivar Kaly), *Lycopersicon esculentum* L. (tomato; 30- to 40-cm stage, cultivar Floradel), *Saintpaulia ionantha* H. Wendl (3 months old; cultivar Blue Rhapsody), *Dieffenbachia exotica* (dieffenbachia; 6- to 8-leaf stage, cultivar Mariane), *Syngonium podophyllum* (syngonium; 6- to 8-leaf stage, cultivar White Butterfly), and *Cichorium intybus* (detached witloof-chicory leaves; cultivar Flash).

Two leaves each from tobacco, dieffenbachia, syngonium, and saintpaulia plants were infiltrated, in three different areas of the parenchymatous tissues, with 200 μl of bacterial suspensions ($10^6$ and $10^8$ CFU $ml^{-1}$). For the negative tobacco results, the injections were repeated with a concentration of $1 \times 10^9$ to $5 \times 10^9$ CFU $ml^{-1}$. For the two tomato plants, inoculations were made by stem puncture. All control plants were similarly inoculated with sterile PS. After inoculation, plants were kept in a growth cabinet under conditions of saturated humidity; day and night temperatures of 28 and 18°C, respectively; and a day length period of 12 h of light.

Two witloof-chicory leaves were prick inoculated in the middle of the leaves (adaxial side) with 30 μl of bacterial suspensions of $10^6$ and $10^8$ CFU $ml^{-1}$ and incubated in a saturated humid chamber at 25°C for 24 and 48 h, respectively. Inoculation with sterile PS was used as a control.

Unrooted carnation cuttings inoculated by standing overnight in $10^6$-CFU (vol/vol) $ml^{-1}$ bacterial suspensions were rooted in trays with perlite for 1 month in the greenhouse at 20°C and were then grown in trays with a mixture of peat and vermiculite in the greenhouse at the same temperature.

Disease incidence records were taken for 4 months after transplanting for carnation, 1 month for diffenbachia and syngonium, 1 week for saintpaulia, and 2 days for the HR on tobacco and witloof-chicory.

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

| Host | Strain[a] | Geographical origin and year isolated | Source[b] | Biovar[c] | rRNA pattern | RFLP group[d] |
|---|---|---|---|---|---|---|
| Banana | 1871 | Ivory Coast, 1976 | 1 | 3 | 44 | 10 |
| Carnation | 1240 (ICPB EC 174) | Denmark, 1956 | 1 | 1 | 29 | ND |
| | 1243 (NCPPB 518) | Denmark, 1957 | 1 | ND | 29 | ND |
| | 1985 | France, 1972 | 1 | 1 | 26 | ND |
| | 2021 | France, 1972 | 1 | 1 | 25 | ND |
| | 795 | France, 1965 | 1 | 1 | 23 | 1 |
| | E II34 | France, 1972 | 2 | 1 | 28 | ND |
| | 1151 | Italy, 1967 | 1 | 1 | 29 | 1 |
| | 30119, 30120, 30121, 30122 | Italy | 3 | 3 | 18 | ND |
| | 1200 (NCPPB 453) | United Kingdom, 1956 | 1 | 1 | 24 | 1 |
| | PD 863 (NCPPB 393) | United Kingdom, 1956 | 4 | 1 | 29 | ND |
| | 1441 | United States | 1 | 5 | 3 | 5 |
| Chicory | 3262 | France, 1981 | 2 | 5 | 1 | ND |
| | SF18-538 | Switzerland | 2 | 7 | 24 | ND |
| Chrysanthemum | 1346 (ICPB EC 239) | Italy | 1 | 5 | 1 | ND |
| | 2048 (NCPPB 402) | United States, 1956 | 1 | 5 | 2 | 5 |
| | 1242 (NCPPB 427) | United States | 1 | 5 | 2 | ND |
| Corn | 1522 | Colombia, 1973 | 1 | 3 | 32 | 7 |
| | 1271 (NCPPB 1065) | Egypt, 1961 | 1 | 3 | 36 | 7 |
| | 1596 | France, 1974 | 1 | 8 | 42 | 8 |
| | 1499 | France, 1973 | 1 | 3 | 34 | 7 |
| | 1528 (NCPPB 2541) | United States, 1966 | 1 | 8 | 40 | ND |
| | 1529 | United States | 1 | 3 | 39 | ND |
| | 1268 (NCPPB 1851) | United States, 1966 | 1 | 3 | 33 | 7 |
| | 2052 (NCPPB 2538) | United States, 1970 | 1 | 3 | 41 | 7 |
| | 1534 | Zimbabwe, 1955 | 1 | 3 | 35 | ND |
| | 2595 | Kenya | 1 | 3 | 37 | ND |
| Dahlia | 3367 | France, 1977 | 3 | 1 | 25 | 1 |
| | 2013 | France, 1974 | 1 | 1 | 26 | 1 |
| Dieffenbachia | 30608 | France, 1974 | 3 | 3 | 5 | ND |
| | 3642 | France, 1974 | 3 | 3 | 10 | 6 |
| | 3665 | France, 1974 | 3 | 2 | 7 | 2 |
| | 2014 | France, 1974 | 1 | 3 | 5 | 6 |
| | 1237 (NCPPB 1514) | Germany, 1962 | 1 | 2 | 7 | 2 |
| | 1152 | Italy, 1968 | 1 | 2 | 7 | 2 |
| | 1870 | Ivory Coast, 1976 | 1 | 2 | 7 | 2 |
| | ED1 | Martinique, 1987 | 5 | 3 | 12 | 6 |
| | 2051 (NCPPB 2976) | United States, 1957 | 1 | 2 | 7 | 2 |
| | 1345 (NCPPB 2308) | Italy, 1969 | 1 | 2 | 7 | 2 |
| Kalanchoe | 1805 | Denmark, 1977 | 1 | 9 | 30 | 1 |
| | 3716 | France, 1978 | 3 | 1 | 30 | 1 |
| | 30728, 30732, 30736 | France, 1977 | 3 | 9 | 30 | ND |
| | 30739 | France | 3 | 9 | 30 | ND |
| | 2982 | France, 1987 | 1 | 9 | 30 | ND |
| | 2598 | Switzerland, 1982 | 1 | 9 | 30 | ND |
| Parthenium | 1270 (NCPPB 516) | Denmark, 1957 | 1 | 6 | 4 | ND |
| | 1236 (NCPPB 1861) | United States, 1945 | 1 | 6 | 4 | ND |
| Pelargonium | 1269 (NCPPB 898) | Comoro Islands, 1960 | 1 | 3 | 21 | 3 |
| Philodendron | 3805 | France | 3 | 3 | 6 | 6 |
| | EP2 | Martinique, 1987 | 5 | 3 | 12 | 6 |
| | PD 471 | The Netherlands | 2 | 3 | 20 | ND |
| | 1248 (NCPPB 454) | United States, 1957 | 1 | 3 | 20 | ND |
| | 1245 (NCPPB 204) | United States, 1959 | 1 | 3 | 20 | 6 |
| Pineapple | 1271 (NCPPB 1125) | Malaysia, 1961 | 1 | 8 | 43 | ND |
| | 1278 (NCPPB 1121) | Malaysia, 1961 | 1 | 3 | 43 | ND |

*Continued on following page*

TABLE 1—*Continued*

| Host | Strain[a] | Geographical origin and year isolated | Source[b] | Biovar[c] | rRNA pattern | RFLP group[d] |
|---|---|---|---|---|---|---|
| Potato | 2267 (NCPPB 3346) | Australia, 1978 | 1 | 3 | 36 | 8 |
| | 2711 (DAR 305-14) | Australia | 1 | 3 | 31 | ND |
| | 1888 (NCPPB 3344) | France, 1978 | 1 | 1 | 22 | 1 |
| | 2288 | France, 1980 | 1 | 1 | 25 | 1 |
| | 2015 (NCPPB 3345) | France, 1975 | 1 | 7 | 27 | 1 |
| | 2593 (CIP 004) | Peru | 1 | 3 | 18 | ND |
| | 2594 | Peru | 1 | 3 | 36 | ND |
| | Cip 366 | Peru | 6 | 3 | 14 | 3 |
| Saintpaulia | 30909, 30913 | France, 1974 | 3 | 3 | 17 | ND |
| | 3937 | France, 1977 | 3 | 3 | 17 | 3 |
| | 30932 | France, 1983 | 3 | 3 | 17 | ND |
| Tobacco | SH230-C94 | Cuba | 2 | 3 | 38 | ND |
| | 1891 | United States | 1 | 3 | 19 | ND |
| Tomato | G3 | Guadeloupe, 1988 | 5 | 3 | 13 | 9 |
| | 722 | France, 1965 | 1 | 1 | 26 | 1 |
| | SA86-10 | France, 1981 | 2 | 5 | 1 | ND |
| | ET1 | Martinique, 1987 | 5 | 3 | 8 | 3 |
| | ET2 | Martinique, 1987 | 5 | 3 | 15 | 3 |
| | ET3 | Martinique, 1987 | 5 | 3 | 11 | 6 |
| | ET5 | Martinique, 1987 | 5 | 3 | 11 | ND |
| | ET11 | Martinique, 1988 | 5 | 3 | 16 | ND |
| | SH230-C143 | Cuba | 2 | 2 | 9 | ND |

[a] Abbreviations: ICPB, International Collection of Phytopathogenic Bacteria, University of California, Davis; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

[b] Coded culture sources: 1, Collection Française de Bactéries Phytopathogènes (CFBP), 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 (31) and Boccara et al. (3).

[d] As determined by Boccara et al. (3).

## RESULTS AND DISCUSSION

*Eco*RI enzyme was previously used to classify clinical (21) and phytopathogenic (2, 35) bacteria with the 16S and 23S rRNA probe from *E. coli*. This enzyme was used for our study. In order to compare the efficiency of ribotyping discrimination with those of other molecular tools, most of our strains were chosen from those previously studied by Boccara et al. (3) (Table 1).

**rRNA restriction patterns.** Forty-four restriction patterns were observed for the *E. chrysanthemi* strains. These are represented schematically in Fig. 1. The pattern number associated with each strain is given in Table 1. For a given strain, the number of fragments that hybridized with the rRNA probe varied from 9 to 16. Different patterns with 8 to 13 fragments have been obtained for *E. carotovora* (44, and unpublished data), while only 4 to 7 fragments have been detected in *Pseudomonas* and *Xanthomonas* spp. (2). Generally, members of the *Enterobacteriaceae* yielded more restriction rRNA fragments than other bacteria, probably because of their greater number of rRNA copies (20).

A total of 47 fragments were thus considered for the phenetic and cladistic analyses. One fragment with an estimated size of 1.3 kbp was observed in 42 patterns from *E. chrysanthemi* and in the 20 strains of *E. carotovora* tested (data not shown). This band seems to be characteristic of *Erwinia* spp. and hence might be of taxonomic value. Commonly obtained patterns are given in Fig. 2.

The phenetic tree obtained using the distance ($D$) $D = 1 - S$ of the Sorensen-Dice similarity coefficient ($S$) and unweighted average pair group clustering method of a whole data set is presented in the dendrogram of Fig. 3. All the trees obtained with the Nei and Li distance or other similarity coefficients and other clustering systems showed very similar topologies (data not shown). Changes were noted only for the location of some leaves in the same cluster and for the value of the genetic distance. Cluster definitions for a distance of 0.7 allowed the separation of strains of *E. chrysanthemi* into seven groups. Finally, cladistic analyses (MIX and CONSENSE programs) grouped the strains in a similar way, with a slight change of some leaves into the same stem, while pattern 44 (banana strain) was included in cluster 5 (corn strains).

**Correlation with pathovars.** To assess the correlation between patterns of rRNA and the differentiation at the intraspecific level of *E. chrysanthemi*, the observed RFLP clusters were compared with the pathovar groupings of this organism (11, 13, 43). The rRNA gene restriction clusters appear to correlate well with the pathovars and botanical classification (Fig. 3).

Cluster 1 contained strains isolated from chrysanthemum, tomato, sunflower, and carnation and corresponds to pv. chrysanthemi. Similar groupings of the same strains have been obtained by other authors (3, 11, 31). Furthermore, cluster 2 contains two strains isolated from parthenium which belong to pv. parthenii. Thus, clusters 1 and 2 might be characteristic of strains from the botanical family related to the *Compositae* (10).

Strains belonging to pv. dieffenbachiae were grouped in cluster 3. Other strains isolated from tropical or subtropical areas also fell into this cluster, which exhibited a high degree of polymorphism. Similarly, Boccara et al. (3), using total DNA restriction digests hybridized with randomly chosen probes and

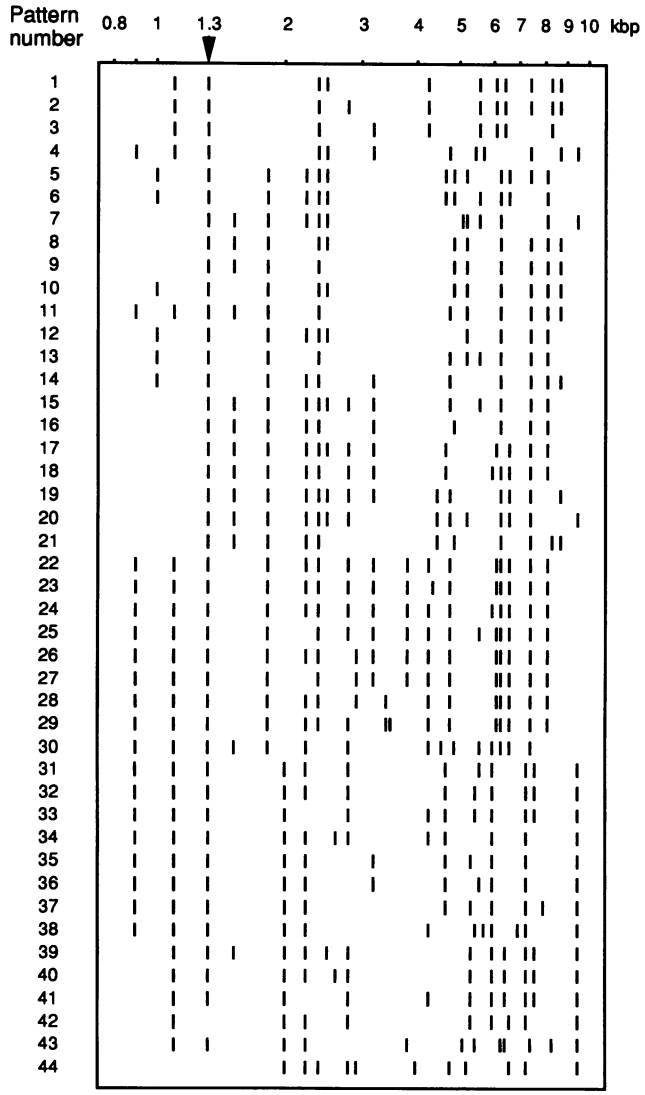


FIG. 1. Schematic representation of the rRNA gene restriction patterns obtained after digestion of total DNAs of some strains of *E. chrysanthemi* with *Eco*RI and hybridization with AAF-labelled rRNA from *E. coli*. Sizes are arranged according to a logarithmic scale and expressed in kilobase pairs.

probes including genes that encode plant cell wall-degrading enzymes, classified 52 strains of *E. chrysanthemi* into 10 RFLP groups. It is interesting to note that our rRNA cluster 3 corresponds to their three related RFLP groups (groups 2, 3, and 6) containing strains isolated from field crops in tropical and subtropical areas or from imported ornamental tropical plants. Furthermore, our results agree with those of Thomson et al. (43). Using 25 nutritional tests, they distinguished strains isolated from philodendron and dieffenbachia, both belonging to the family *Araceae*, into two closely related pathovars, philodendroni and dieffenbachiae, respectively. Unfortunately, carnation strains isolated in Italy and clustered in this group have not been tested by other workers to allow comparison with our results. This cluster grouped strains isolated from plants (tomato, tobacco, potato, and saintpaulia) of the *Asteridae* subclass (10) as in the clusters 1 and 2.

Cluster 4 formed a very homogeneous group of strains

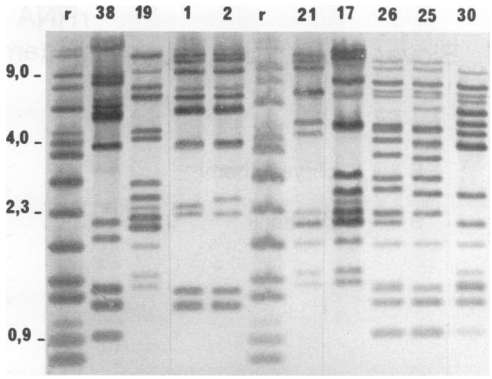


FIG. 2. Southern blot of *Eco*RI-digested total DNAs of *E. chrysanthemi* strains hybridized with AAF-labelled rRNA from *E. coli*. Lanes r, molecular size standard Raoul I; other lane numbers correspond to pattern numbers given in Table 1.

isolated from field crops or ornamental plants and corresponded to pv. dianthicola. Our association (in this cluster) of the strains isolated from carnation and other hosts (as the strain 722 isolated from tomato) has previously been reported by using biochemical and pathogenicity tests (12) and membrane protein profiles (44).

All kalanchoe strains isolated in Europe are grouped in cluster 4 in the same specific leaf, which is slightly separated from the other leaves of this cluster. Our results agree with those of Boccara et al. (3), who have grouped the strains of this leaf in a very homogeneous RFLP group 1, while other authors find it difficult to identify kalanchoe strains on the basis of biochemical characteristics (31).

The 10 corn strains, differing in their geographical origins and years of isolation, were found to be characterized by 10 different patterns, thus indicating a high degree of polymorphism, but were grouped together in cluster 5, corresponding to pv. zeae. Such heterogeneity was previously reported (23, 43).

The two potato strains from Australia, which were grouped in cluster 5 with the corn strains, have been previously included in pv. zeae (9). Cother and Powell (9) showed that these potato strains are pathogenic in corn and suggested that corn may be a source of contamination to neighboring potato crops. The same may be true of the potato strain from Peru which is also assigned to this cluster. For this reason, these strains were excluded from the correlation analysis of geographical origin. Brenner et al. (6) indicated that strains of *E. chrysanthemi* isolated from corn and grasses were grouped in one group. Thus, we suggest that cluster 5 is probably related to the botanical family *Poaceae*.

Cluster 6 contained strains isolated from pineapple belonging to pv. zeae (11). This cluster is very close to clusters 5 and 7, and all three of the clusters contain strains isolated from monocotyledoneous plants of the *Commelinidae* subclass (10).

Cluster 7 consists of a single strain (strain 1871) isolated in the Ivory Coast from *Musa* sp. and thus might be representative of the pv. paradisiaca. Another banana strain (strain 1445 CFBP) isolated in Colombia was also studied but gave an rRNA pattern different from those of the *E. chrysanthemi* strains (data not shown). Moreover, the DNA of this last strain was not amplified by PCR with primers specific for *E. chrysanthemi* strains (unpublished data). Similarly, Ngwira (31) classified this atypical banana strain in a different biovar, biovar 4, and Boccara et al. (3) showed that, together with other banana

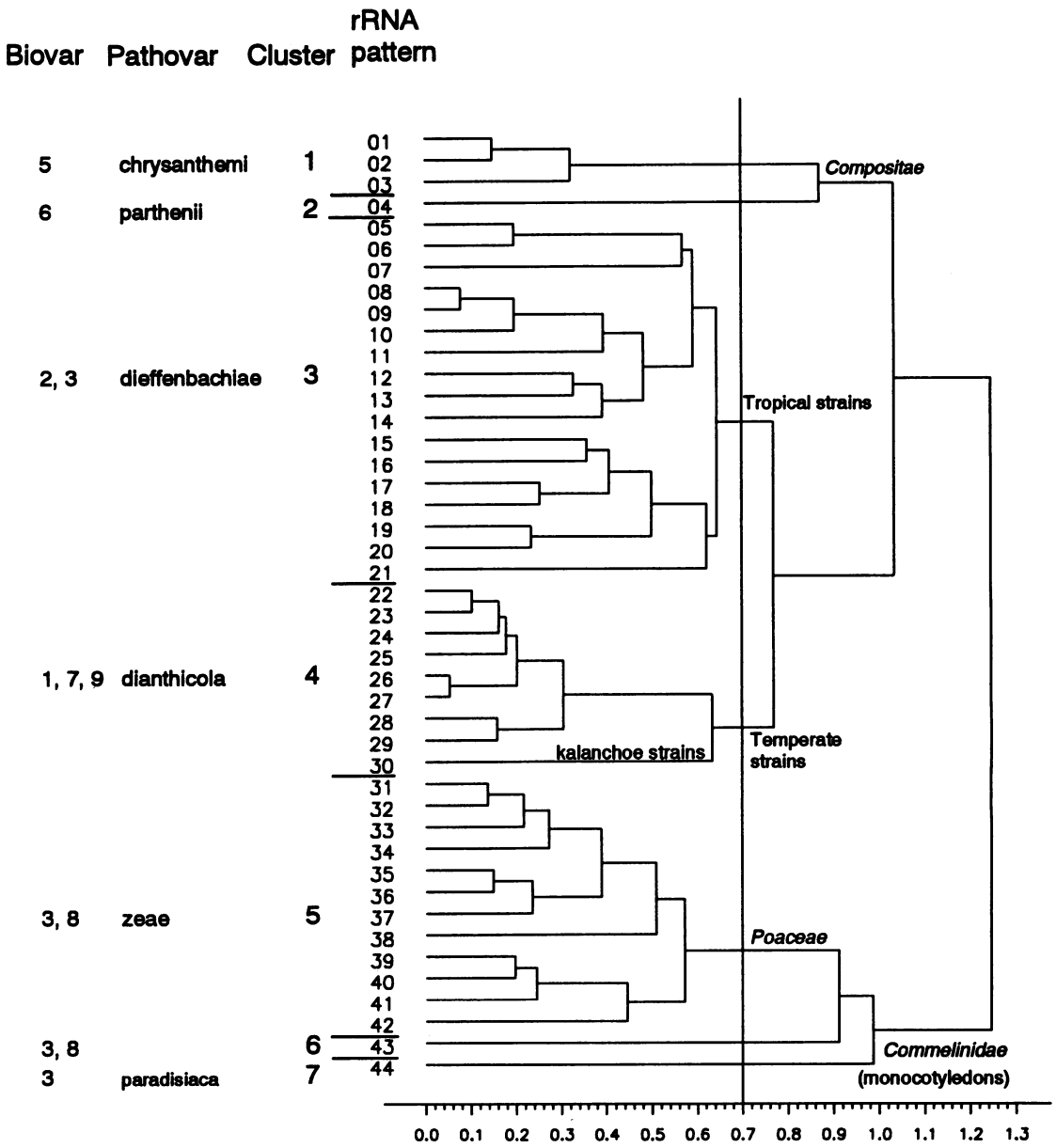


FIG. 3. Dendrogram derived from the unweighted average pair group cluster of the distance $D = 1 - S$, with $S$ as the Sorensen-Dice similarity coefficients of the patterns schematically presented in Fig. 1. Numbers refer to the rRNA pattern numbers given in Table 1.

strains from Colombia and the French West Indies, it fell into separate RFLP group 4 and PL group 2 and did not hybridize to their pMH18 probe including the *celY* gene. It is thus highly questionable whether this strain is an *E. chrysanthemi* strain.

Our results show a good relationship of rRNA restriction pattern clustering with pathovar divisions, at least for five clusters (clusters 1, 2, 4, 5, and 7). Despite the small number of strains grouped in clusters 2, 6, and 7, which contain strains from parthenium, pineapple, and banana, respectively, the cluster groupings obtained suggest that a relationship between rRNA pattern and host of origin but mainly botanical taxonomy exists. However, the results of this study also indicate that heterogeneity within some clusters might be related to the broad host range of strains of tropical and subtropical areas. For example, greater heterogeneity was noted in cluster 3 compared with cluster 4. The subdivision of cluster 3 into three to four subgroups is questionable. Other authors have also shown that there is greater variation among pathovars with a wide host range than among those with a narrow host range (2, 11).

**Correlation with biovars.** When the rRNA clusters were compared with the nine biovars previously described (30, 39), they were found to relate strongly to the biovar divisions. For example, cluster 1 corresponds to biovar 5, cluster 2 corresponds to biovar 6, cluster 4 corresponds to biovar 1, and cluster 5 corresponds to biovar 3. Moreover, clusters 5, 6, and 7, which group strains isolated from *Commelinidae* plants, were all strains of biovars 3 and 8, two closely related biovars (31).

Strains from cluster 3 belong to biovars 2 and 3; biovar 2 contains dieffenbachia strains, and biovar 3 contains strains

TABLE 2. Reactions of several plants to strains of *E. chrysanthemi* isolated from different hosts

| Cluster | Bacterium strain | Host of origin | Reaction[a] of inoculated: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Chicory | Tobacco | Dieffenbachia | Syngonium | Saintpaulia | Tomato | Carnation |
| 1 | 1441 | Carnation | M | HR | m | m | M | – | M |
| 1 | 2048 | Chrysanthemum | M | HR | – | – | M | M | M |
| 3 | 30121 | Carnation | M | HR | m | M | M | – | w |
| 3 | Cip366 | Potato | M | HR | m | M | M | – | – |
| 3 | 3937 | Saintpaulia | M | HR | – | – | M | – | – |
| 3 | ET3 | Tomato | M | HR | M | M | M | – | 0 |
| 3 | EP2 | Philodendron | M | HR | M | M | M | – | 0 |
| 3 | ED1 | Dieffenbachia | M | HR | M | M | M | – | 0 |
| 4 | 1985 | Carnation | – | – | – | – | m | W | W |
| 4 | 1888 | Potato | – | – | m | – | M | W | W |
| 4 | 3367 | Dahlia | – | – | – | – | – | W | W |
| 4 | 3716 | Kalanchoe | m | – | m | m | M | W | W |

[a] M, maceration; W, wilting and stunting; m and w, less-virulent symptoms; HR, hypersensitivity reaction; –, no symptom; 0, tests not effectuated.

essentially isolated from tropical ornamental hosts (23) or from different plants in tropical areas (31).

In cluster 4 are found identical strains or those with closely related rRNA patterns belonging to biovars 1, 7, and 9. These three biovars appear to be similar (38), in particular, in their growth requirements (31). In agreement with our analysis, Boccara et al. (3) grouped the same strains from these three biovars in their RFLP group 1.

The strains of cluster 5 were distributed into biovars 3 and 8, which are separated by only one biochemical test (30). These results are consistent with those of Janse and Ruissen (23), who showed that all their corn strains belong to biovar 3. On the other hand, strains included in biovar 3 were separated by our results into clusters 3, 5, 6, and 7, suggesting that rRNA grouping may be more discriminative than biovar analysis. Thus, as suggested by R. Samson (38), biovar 3, the largest, should be further subdivided.

**Correlation with geographical origin. (i) Correlation at the cluster level.** Strains belonging to clusters 1 and 4 were isolated from diverse hosts in the temperate regions. Cluster 3 contains strains isolated from field crops in tropical or subtropical areas or in temperate countries from ornamental plants imported from tropical or subtropical regions generally grown in greenhouses.

As reported above for pathovar differences, strains from cluster 4 form a very homogeneous group that agrees with results of other workers who showed that several strains which belong to this group are unable to grow at 39°C (3, 31). This characteristic might be related to adaptation to crops grown in temperate climates, according to Janse and Spit (24), who named them cold strains.

However, this kind of grouping which is related to geographical origins is not obvious for all our strains. In particular, corn strains from different geographical areas were grouped in the same cluster 5 that might result from a monophyletic origin. This is in contrast to the findings of other workers (3, 31).

**(ii) Correlation at the host plant level.** Strains from different geographical regions isolated from the same host plant, tomato, potato, or carnation, were analyzed. The main difficulty in this analysis is that these strains are not fully characterized for their pathogenicity traits. Although they were isolated from these plants, we do not know whether they are pathogenic or only epiphytic on their respective hosts of origin.

The nine tomato strains were distributed into clusters 1, 3, and 4. The seven strains from cluster 3 came from tropical and subtropical countries, while the two strains of clusters 1 and 4 came from France (temperate country). The two French strains were previously reported to belong to two different biovars (31), which suggests that they are not specific to tomato.

Potato strains were grouped in clusters 3 and 4 according to their geographical origins, namely, tropical countries and France, respectively. These strains were also analyzed by Boccara et al. (3), who grouped them in 2 RFLP groups, namely, groups 1 and 3. Similarly, other investigators grouped strains of *E. chrysanthemi* from potato in different groups (23, 31).

In the case of carnation strains, an inconsistent relationship between cluster and geographical region (e.g., Italian strains of cluster 3 compared with the other European strains) which might be attributed to the commercial distribution of plant was found.

**Correlation with pathogenicity.** Responses of tobacco, tomato, dieffenbachia, syngonium, saintpaulia, carnation, and detached leaves of witloof-chicory to inoculations with strains belonging to selected clusters are summarized in Table 2.

Eleven of the 12 tested strains macerated saintpaulia tissue to various degrees. The carnation cuttings inoculated with the two American carnation or chrysanthemum strains of cluster 1 were severely macerated and died before rooting. Moreover, these strains showed vigorous pectic enzyme activity in witloof-chicory detached leaves and induced HR in tobacco. The American chrysanthemum and carnation strains were previously reported to produce the same isozymes and to belong to PL group 4 (3).

Strains belonging to cluster 3 were generally unable to produce symptoms in tomato or carnation plants. However, the Italian carnation strain included in this cluster produced typical symptoms in carnation plants but seemed to be less virulent than those of cluster 4. On the other hand, all the strains produced strong maceration symptoms in witloof-chicory, dieffenbachia, and syngonium plants and an HR reaction in tobacco. These strains belong to the biovar 3, characterized as strongly pectinolytic (23), and produced the five PL isozymes (3) and hence may be capable of infecting a wider host range, producing various symptoms.

All strains belonging to cluster 4 were capable of colonizing carnation and tomato stems, producing wilting and stunting symptoms. This result suggests that although polymorphic, these strains are true members of pv. dianthicola, which is

characterized by the ability to produce wilting in carnations (12). On the other hand, these strains were unable to macerate witloof-chicory, indicating their weak or moderate PL production in a PL-inducing medium (unpublished data) as previously stated by Janse and Ruissen (23) for biovar 1. Furthermore, Ried and Collmer (36) suggested a possible correlation between *E. chrysanthemi* PL isozyme profiles and their original host. They demonstrated that carnation strains produce in vitro only 2 PL isozymes, one neutral and one basic. This might explain their weak maceration activity. Cluster 4 generally gave negative reactions in other hosts, except the kalanchoe strain, which produced weak maceration in saintpaulia, dieffenbachia, and syngonium plants. This kalanchoe strain was distantly related to the other strains of this cluster and seems to have intermediate characteristics between the strains of this cluster and those of clusters 3 and 4, possessing greater pectic enzyme activity.

Under our conditions, HR reaction was induced by all the strains of clusters 3 (pv. dieffenbachiae and philodendroni and biovar 3) and 1 (pv. chrysanthemi and biovar 5) but not by the strains of cluster 4 (pv. dianthicola and biovar 1). Thus, all the strains inducing tobacco HR are also able to highly macerate chicory leaves. However, the necrosis symptoms observed with strains of clusters 1 and 3 could be due to the *hrp* genes, the presence of which in *E. chrysanthemi* was recently demonstrated by Bauer and Collmer (1). In this case it is intriguing to observe the absence of an HR reaction for the strains of cluster 4, even at a concentration of $5 \times 10^9$ CFU $ml^{-1}$. Such an absence of an HR reaction has also been obtained for several subspecies of *E. carotovora* (35) and for *Erwinia stewartii*, an $HR^-$ bacterium with *wtsA*, an *hrp*-related gene (17). The *hrp* gene expression has been shown to be highly dependent on the growth conditions and to be affected by plant components (46). Such a regulation may thus differ between strains. Moreover, the homology between the regulatory genes *hrpS* and *wtsA* (17) and *nifA* and *ntrC* (19), although the latter three genes do not induce HR, may indicate the complexity of regulation of the *hrp* genes of *Erwinia* spp. by the plant components. A differential regulation of the *hrp* genes between *E. chrysanthemi* strains might thus explain the difference we observed between the several clusters.

Our results show for the first time a clear distinction between strains pathogenic to monocotyledons (clusters 5 to 7) and those pathogenic to dicotyledons (clusters 1 to 4). Only the distribution of the six strains isolated from members of the family *Arecidae* (philodendron and dieffenbachia) remains to be explained. The rRNA patterns are highly correlated with the botanical classification, with the pathovar division, and to a lesser extent, with the geographic origin of the strains. Furthermore, strains of several clusters correlated mostly with the biovar classification. In the case of biovar 3, our results suggest that subdivision of the biovar is necessary.

However, the *E. chrysanthemi* species appears as highly polymorphic, and we were unable to define, under our RFLP conditions, an rRNA pattern typical for each pathovar (e.g., pathovar zeae). This high polymorphism observed within a pathovar, i.e., a cluster, might be related to the large number of hosts of origin of the strains we studied. It may be a result of the wide host range of this species (more than 50 hosts can be infected) (4) and may indicate that further subdivisions of the pathovars are also necessary.

Our cluster divisions seem to show that a certain host specificity may exist. Particularly, we have shown that related strains, within the same cluster, induce similar symptoms in the plants.

In general, highly pectinolytic strains of *E. chrysanthemi* seem quite ubiquitous and capable of macerating a broad range of host plants. Compared with more specific pathogenicity tests, such as carnation wilting, the maceration capacity of strains appears to be a more general symptom that is less correlated to host specificity.

Provided ribotyping is applied to a larger number of strains, this analysis technique appears to be an accurate, useful tool, in particular for taxonomic and epidemiological studies. This method should allow the clear definition of groups that may further solve the controversial question of host specificity in *E. chrysanthemi*.

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