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PCR and Restriction Fragment Length Polymorphism of a *pel* Gene as a Tool To Identify *Erwinia carotovora* in Relation to Potato Diseases

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Using a sequenced pectate lyase-encoding gene (*pel* gene), we developed a PCR test for *Erwinia carotovora*. A set of primers allowed the amplification of a 434-bp fragment in *E. carotovora* strains. Among the 89 *E. carotovora* strains tested, only the *Erwinia carotovora* subsp. *betavasculorum* strains were not detected. A restriction fragment length polymorphism (RFLP) study was undertaken on the amplified fragment with seven endonucleases. The *Sau*3AI digestion pattern specifically identified the *Erwinia carotovora* subsp. *atroseptica* strains, and the whole set of data identified the *Erwinia carotovora* subsp. *wasabiae* strains. However, *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *odorifera* could not be separated. Phenetic and phylogenic analyses of RFLP results showed *E. carotovora* subsp. *atroseptica* as a homogeneous group while *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera* strains exhibited a genetic diversity that may result from a nonmonophyletic origin. The use of RFLP on amplified fragments in epidemiology and for diagnosis is discussed.

Pectolytic erwinias cause diseases on a wide range of plants. The species *Erwinia carotovora* is divided into the four subspecies *atroseptica*, *carotovora*, *betavasculorum*, and *wasabiae* (13, 20). *Erwinia carotovora* subsp. *betavasculorum* and *Erwinia carotovora* subsp. *wasabiae* cause soft rot of sugar beet and Japanese horseradish, respectively (13, 37). *Erwinia carotovora* subsp. *atroseptica* is usually restricted to potato under cool temperate climate (27), while *Erwinia carotovora* subsp. *carotovora* seems to be more ubiquitous. Some strains, not isolated from potato, have been described as atypical *E. carotovora* subsp. *atroseptica* because they exhibit, for example, the ability to grow at 37°C (24, 29). A new subspecies called *Erwinia carotovora* subsp. *odorifera* was proposed for the atypical strains isolated from several hosts, including chicory, that produce odorous volatile metabolites (12).

Because of their economic importance, we oriented our study on potato diseases. Symptoms on potato include blanking, blackleg, wilting, and soft rotting of aerial stem, tuber, and stolon end (28). *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* cause these symptoms under certain field conditions and are found as mixed populations with differential proportions depending on environmental conditions (28, 34). *E. carotovora* subsp. *atroseptica* is considered the typical blackleg causal agent in Europe, particularly at the beginning of the growing season. Conversely, in pathogenicity tests on potato plants, most *E. carotovora* subsp. *carotovora* strains do not produce a typical blackleg symptom at low temperatures (29). However, *E. carotovora* subsp. *carotovora* is associated with blackleg of potato in Arizona and Colorado (35). Therefore, it could be interesting to develop identification tests useful in diagnosis.

Classical techniques of bacterial isolation and characterization are time-consuming and relatively insensitive because of the high level of saprophytes in the samples (21). Development of selective media like CVP (crystal violet pectate) minimizes the problem of sensitivity (3). Immunological techniques have been developed, and their efficiency is increased by culture on selective medium (16), but the serological tests are complicated because of the high number of serogroups within *E. carotovora* (6). New molecular tools like hybridization probes have been demonstrated to be very efficient in plant disease diagnosis (26), and some probes have been developed for pectolytic erwinias (40). However, the use of such probes for routine tests is limited by the relatively low signal of the nonradioactive labelling. Enrichment of the sample in growth medium improves sensitivity, but remains time-consuming (40). PCR enhances the sensitivity of detection by the in vitro amplification of defined DNA target sequences (32, 36).

Pectate lyases are involved in soft-rot diseases. Several *pel* genes (encoding pectate lyases) were sequenced, and three families were identified, on the basis of high sequence homology (14). The first family (*BC* family) contains *pel* genes that are common to *Erwinia chrysanthemi* and *E. carotovora*, the second family consists of genes present only in *E. chrysanthemi* (*ADE* family), and the third group corresponds to genes found in some strains of *E. carotovora* and in the bacterium *Yersinia pseudotuberculosis* (*Y* family) (14, 23, 38). In this study, we used *pel* genes from the *Y* family, to identify *E. carotovora* species and subspecies. Phenetic and phylogenic relationships between the strains were established from the restriction fragment length polymorphism (RFLP) results. A patent concerning this work has been filed (4a).

MATERIALS AND METHODS

Bacterial strains. Pectinolytic *E. carotovora* strains are described in Table 1. Other *Erwinia* species including *Erwinia amylovora* (1 strain), *Erwinia herbicola* (2 strains), *Erwinia rhapontici* (1 strain), and pectolytic *E. chrysanthemi* (32 strains) were also tested. Furthermore, our collection contains pectolytic bacteria associated with soft rot symptoms (number of

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TABLE 1. Origins and RFLP groups of *E. carotovora* strains used in PCR-RFLP study

Strain	Isolated from:	Country and yr [a]	RFLP group[b]
Erwinia carotovora subsp. *atroseptica*			
88.33	Potato	France, 1988[c]	1
88.45	Potato	France, 1988[c]	1
88.1	Potato	France, 1988[c]	2
88.22a	Potato	France, 1988[c]	1
88.24	Potato	France, 1988[c]	1
88.30a	Potato	France, 1988[c]	1
87.7	Potato	France, 1987[c]	2
87.13	Potato	France, 1987[c]	1
87.16a	Potato	France, 1987[c]	1
87.16b	Potato	France, 1987[c]	1
86.14.11	Potato	France, 1986[c]	1
86.20	Potato	France, 1986[c]	1
511	Potato	France, 1964[d]	1
SF1.1	Potato	Germany[e]	1
161	Potato	The Netherlands[f]	1
CIP114	Potato	Peru, 1980[g]	1
CIP125	Potato	Peru, 1980[g]	1
CIP131	Potato	Peru, 1980[g]	1
CIP026	Potato	Peru, 1980[g]	2
SH164.4	Potato	La Réunion, 1988[c]	1
CH3	Potato	Switzerland, 1985[h]	2
CH5	Potato	Switzerland, 1985[h]	1
CH6	Potato	Switzerland, 1985[h]	1
SF18.296	Potato	Switzerland, 1958[c]	1
1329	Potato	UK, 1967[d]	1
1330	Potato	UK, 1967[d]	2
SCRI1043	Potato	UK, 1985[i]	1
1526	Potato	UK, 1957[d]	1
1527	Potato	USA, 1973[d]	1
1525	Potato	USA, 1969[d]	1
1453	Tomato	France, 1973[d]	2
1546	Tomato	France, 1973[d]	2
Erwinia carotovora subsp. *betavasculorum*			
SF142.2	Artichoke	La Réunion, 1986[c]	Not amplified
2121	Sugar beet	USA, 1972[d]	Not amplified
2122	Sugar beet	USA, 1971[d]	Not amplified
1520	Sunflower	Mexico[d]	Not amplified
Erwinia carotovora subsp. *carotovora*			
89.19[j]	Potato	Argentina, 1989[c]	8
1H[k]	Water	Spain, 1989[l]	8
40H[k]	Water	Spain, 1989[l]	8
SH230.134[m]	Banana	Cuba[c]	19
CM1[m]	Cabbage	Malawi, 1986[c]	17
798	Carrot	USA[d] (ATCC 495)	9
CH15	Celery	Switzerland, 1988[h]	9
1489	Chrysanthemum	France, 1971[d]	4
1458[m]	Chrysanthemum	USA, 1971[d]	8
SH230.115[m]	Corn	Cuba[c]	19
1350[m]	Cucumber	Italy[d]	8
1285	Cyclamen	Greece[d]	6
SE99.1	Witloof chicory	France, 1985[c]	8
1488	Iris	France, 1973[d]	9
SB89.7	Leek	France, 1982[c]	9
2046[T][n]	Potato	Denmark, 1952[d]	12
88.22c	Potato	France, 1988[c]	9
88.29a1[o]	Potato	France, 1988[c]	6
88.44	Potato	France, 1988[c]	4
87.25	Potato	France, 1987[c]	10
86.14.51	Potato	France, 1986[c]	9
S99	Potato	France, 1977[c]	18
S101	Potato	France, 1977[c]	4
76.26	Potato	France, 1976[c]	9
PM2	Potato	Malawi, 1986[c]	15
194	Potato	Morocco, 1963[d]	11
CIP360[m]	Potato	Peru, 1984[g]	13
CIP361[m]	Potato	Peru, 1984[g]	13
CIP009[o,p]	Potato	Peru, 1977[g]	5

Continued on following page

TABLE 1—*Continued*

Strain	Isolated from:	Country and yr[a]	RFLP group[b]
CH24	Potato	Switzerland, 1987[h]	14
CH26	Potato	Switzerland, 1985[h]	4
SCRI193	Potato	USA[i]	14
1336	Potato	UK, 1967[d]	11
SI82.1[m]	Potato	Vietnam, 1989[e]	8
SG162.6[p]	Sunflower	France, 1987[e]	8
1403[p]	Sunflower	Yugoslavia, 1969[d]	16
797[m]	Tobacco	USA, 1951[d]	13
SG39.1[p]	Unknown	La Réunion, 1987[e]	20
SG39.3[m]	Unknown	La Réunion, 1987[e]	8
E. carotovora subsp. *odorifera*			
1893	Celery	France, 1976[d]	3
CH11	Celery	Switzerland, 1985[h]	7
2155	Witloof chicory	France, 1983[d]	5
2154	Witloof chicory	France, 1982[d]	4
1892	Witloof chicory	France, 1981[d]	6
1959	Witloof chicory	France, 1980[d]	4
1878	Witloof chicory	France, 1979[d]	4
1879	Witloof chicory	France, 1979[d]	5
1880	Witloof chicory	France, 1979[d]	3
1646.2	Leek	France, 1980[e]	3
1654	Leek	France, 1980[e]	3
CH4	Lettuce	Switzerland, 1986[h]	4
E. carotovora subsp. *wasabiae*			
3304	*Eutrema wasabi*	Japan[d]	21
3308	*Eutrema wasabi*	Japan[d]	21

[a] UK, United Kingdom; USA, United States of America.

[b] Numbers (1 to 21) of RFLP groups correspond to the restriction maps (I to XVII) of Fig. 2: 1, IV, VII, X, XV; 2, IV, VI, XII, XVII; 3, I, VI, XII, XVI; 4, I, VIII, XII, XVI; 5, I, VI, X, XV; 6, I, V, X, XV; 7, I, VI, X, XVI; 8, II, VI, X, XV; 9, I, V, XII, XV; 10, II, VI, XII, XV; 11, III, V, XII, XV; 12, I, VIII, XII, XV; 13, II, VIII, X, XV; 14, I, V, XII, XVI; 15, II, V, XIV, XV; 16, I, VIII, X, XVI; 17, II, VII, XI, XV; 18, I, VIII, XIII, XV; 19, III, IX, X, XV; 20, III, VIII, XII, XV; 21, III, VII, X, XV.

[c] Bernard Jouan, Institut National de la Recherche Agronomique, Rennes, France, personal collection.

[d] Collection Française de Bactéries Phytopathogènes, Angers, France.

[e] Régine Samson, Institut National de la Recherche Agronomique, Angers, France, personal collection.

[f] Research Institute for Plant Protection, Wageningen, The Netherlands.

[g] International Potato Center, Lima, Peru.

[h] Olivier Cazelles, Station Fédérale de Recherches Agronomiques, Changins, Switzerland, personal collection.

[i] Scottish Crop Research Institute, Invergowrie, Dundee, United Kingdom.

[j] Atypical *E. carotovora* subsp. *atroseptica* strain able to grow at 37°C, exhibiting pathogenicity traits of *E. carotovora* subsp. *carotovora* strains (29).

[k] Atypical *E. carotovora* subsp. *atroseptica* strain able to grow at 37°C, exhibiting pathogenicity traits identical to those of *E. carotovora* subsp. *carotovora* strains (29) and identical in our study to those of six other atypical strains isolated from water in Spain (data not shown).

[l] Maria Lopez, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain, personal collection.

[m] Atypical *E. carotovora* subsp. *carotovora* strain producing indole from tryptophan (29).

[n] Type strain of the species.

[o] Atypical *E. carotovora* subsp. *carotovora* strain, not fully characterized as *E. carotovora* subsp. *carotovora* strain, exhibiting pathogenicity traits of *E. carotovora* subsp. *atroseptica* strains (29).

[p] Atypical *E. carotovora* subsp. *carotovora* strain unable to grow at 37°C (29).

strains in parentheses): *Bacillus polymyxa* (1), *Bacillus pumilus* (1), *Pseudomonas marginalis* (2), *Pseudomonas viridiflava* (1) and *Pseudomonas fluorescens* (2), and other microorganisms, including a pectolytic *Frankia* strain (1), *Pseudomonas solanacearum* (3), *Xanthomonas campestris* (3), *Clavibacter* species (3), *Agrobacterium tumefaciens* (1), nitrogen-fixing bacteria (6), fungi and yeasts (5), potato saprophytes (20), *Y. pseudotuberculosis* (2), *Pseudomonas* species (2), *Comomonas* species (2), and *Enterobacter* species (1). The last five strains were provided by J. van der Wolf (Research Institute for Plant Protection, Wageningen, The Netherlands) as strains cross-reacting with *Erwinia* antibodies.

Chromosomal DNA isolation. Bacteria were grown overnight at 30°C in Luria broth (25). Two milliliters of culture was microcentrifuged, and the DNA was extracted by the method of Klotz and Zimm (17), modified by reducing the volumes by a factor of 10. Integrity of DNA was tested by spectrophotometry and endonuclease restriction (*Eco*RI) followed by electrophoresis in a 0.8% agarose gel (22).

Sequence analysis. The following published sequences were used: *pelY*, *pel153*, *pelB* (14, 23, 38); they were compared with the other *pel* gene sequences available in GenBank and EMBL data bases (releases 75.0 and 33.0, respectively; *Ecapali*, *Ecapalx*, *Ecapela*, *Ecapelb*, *Echpel*, *Echpelb*, *Echpelc*. . .). The primers were chosen in the open reading frames of *pel* genes, after alignment of their sequences by pairs, with programs developed by the University of Wisconsin Genetics Computer Group (8).

DNA amplification. The PCR medium used was that recommended for the *Taq* polymerase from Perkin-Elmer Cetus Corp. (Norwalk, Conn.). The reaction was performed in a 50-μl volume topped with 50 μl of mineral oil (Sigma, St. Louis, Mo.). Samples contained 50 ng of target genomic DNA and 50 pmol of each primer. The mixture was subjected to 25 cycles of the following incubations: 1 min at 94°C, 1 min at 65°C, and 1 min and 30 s at 72°C (Gene ATAQ Controller, Pharmacia LKB, Uppsala, Sweden). Initial analysis of PCR

products was done by electrophoresis in 1% (wt/vol) agarose minigels (22).

Restriction fragment analysis. Forty microliters of PCR samples was ethanol precipitated and resuspended in 20 μl of Tris-EDTA buffer (22). Digestion with *Alu*I, *Hae*II, *Sau*3AI, *Hpa*II, *Taq*I, *Hae*III, and *Hha*I occurred for 1 to 2 h in a 15-μl volume, with 5 μl of DNA solution, according to the supplier's recommendations. The whole sample was electrophoresed on a 6% polyacrylamide gel, with 1 μg of marker V (Boehringer GmbH, Mannheim, Germany) as a size marker. Restriction site positions were determined from fragment size, restriction maps of the sequenced genes, and results from double digestions.

Phenetic and phylogenic data analyses. (i) Cladistic analyses. Digestion enzyme data were analyzed by three methods available from the PHYLIP package (version 3.4) (10, 11). The presence or absence of restriction sites was scored by either a 1 or a 0, respectively. The resulting data matrices were analyzed by the maximum likelihood method with the "restml" program. Ten runs were done with different strain input orders (options U, G, L = 4, and E = 100.0), and the best tree was chosen for its highest log likelihood value. The second approach was a Wagner parsimony method with the "mix" program (options U and P = Wagner). Ten runs were also done with different strain input orders, and the resulting trees were submitted to a "consense" program which writes the consensus tree. The resulting trees were drawn by the Drawtree program (Tree Draw Deck, D. G. Gilbert, Indiana University, 1990).

(ii) Phenetic analysis. Finally, a matrix pair-wise distance ($D = 1 - S$) was calculated from the 0-1 matrix with the Sorensen-Dice similarity coefficient (S) (15), and a hierarchical classification was established by the unweighted average pair-group method of clustering with the Statistical Analysis Software (SAS Institute Inc., Cary, N.C.).

RESULTS

PCR test. We aligned the sequences corresponding to the open reading frames of three genes of the *Y* family: *pel153*, *pelB*, and *pelY*. Conserved regions in *pel* genes of erwinias were retained only when they were different from the *pelY* (from *Y. pseudotuberculosis*) sequence. Another condition was that the distance between the primers was short enough for easy amplification but not so short as to restrict polymorphism analysis in the target region (around 400 to 500 bp). Two oligonucleotides (24-mers) separated by 434 bp were selected as PCR primers, namely, Y_1 (5′TTACCGGACGCCGAGCT GTGGCGT3′) and Y_2 (5′CAGGAAGATGTCGTTATCGC GAGT3′). The sequence of each primer was checked in the complete GenBank and EMBL data bases for identity, allowing up to five mismatches. No homologous sequence was found in these data bases except for the *pel* sequences used in this study.

The primers were used in amplification experiments with six bacterial strains (Fig. 1) to determine their specificity by varying temperature conditions. Our collection of microorganisms was tested for the specificity of this PCR test. An amplified fragment of 434 bp was obtained from all *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *odorifera*, and *E. carotovora* subsp. *wasabiae* strains. No amplified fragment was observed with *E. carotovora* subsp. *betavasculorum*, *E. chrysanthemi*, other *Erwinia* species, and the other microorganisms tested. Thus, the amplification conditions allowed the specific detection of all the strains of pectolytic *E. carotovora*, except the *E. carotovora* subsp. *betavasculorum* strains.

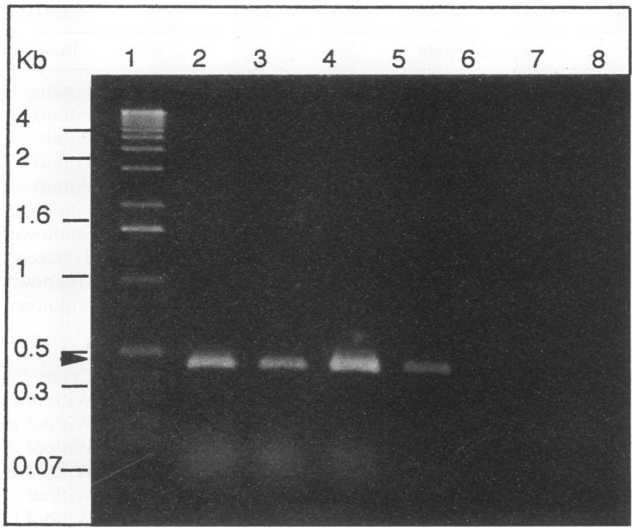

FIG. 1. Agarose electrophoresis of PCR-amplified DNA from several strains. Lanes: 1, 1-kb DNA ladder; 2, *E. carotovora* subsp. *atroseptica* 86.20; 3, *E. carotovora* 1H; 4, *E. carotovora* subsp. *carotovora* SCRI193; 5, *E. carotovora* subsp. *odorifera* 2154; 6, *E. carotovora* subsp. *betavasculorum* 2122; 7, *E. chrysanthemi* 3937 (19); 8, *Y. pseudotuberculosis* ATCC 23208. The arrowhead indicates the 434-bp amplified fragment.

RFLP analysis. An RFLP analysis was undertaken on the collection of *E. carotovora* strains by digesting the 434-bp amplified fragments with several enzymes. *Taq*I and *Hae*III did not give any polymorphism when tested on a representative collection of various strains. Conversely, *Hha*I gave more than 15 different digestion profiles on a range of 30 strains and was not used further. *Alu*I, *Hae*II, *Sau*3AI, and *Hpa*II gave, for the whole collection, three to five different profiles (Fig. 2). Combined results of these four restriction enzymes gave 21 RFLP groups (Table 1).

With the whole set of data, *E. carotovora* subsp. *atroseptica* strains were divided into two subgroups (groups 1 and 2). *E. carotovora* subsp. *wasabiae* strains tested were in one RFLP group (group 21). *E. carotovora* subsp. *carotovora* strains were not clearly distinguished from *E. carotovora* subsp. *odorifera*: some RFLP groups (groups 3 and 7) were specific for *E. carotovora* subsp. *odorifera* strains, but several isolates of *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera* shared the same RFLP group (groups 4, 5, and 6). *E. carotovora* subsp. *carotovora* strains were distributed in 16 different RFLP groups.

Cladistic and phenetic analyses. We compared maximum likelihood ("restml"), Wagner parsimony ("mix"), and genetic distances on the whole set of data. The reliability of the cladistic trees was assessed as described by Debener et al. (5). As a whole, the main partitioning of our strains was conserved and appeared relatively reliable as obtained by these three methods.

The classification obtained by genetic distances (unweighted average pair-group method of clustering) is illustrated in Fig. 3. Three main domains, A, B, and C, were evidenced. The A domain comprised all the *E. carotovora* subsp. *atroseptica* strains and was divided into two subgroups in which all the strains appeared strictly identical. The B cluster contained all the *E. carotovora* subsp. *odorifera* strains and numerous *E.*

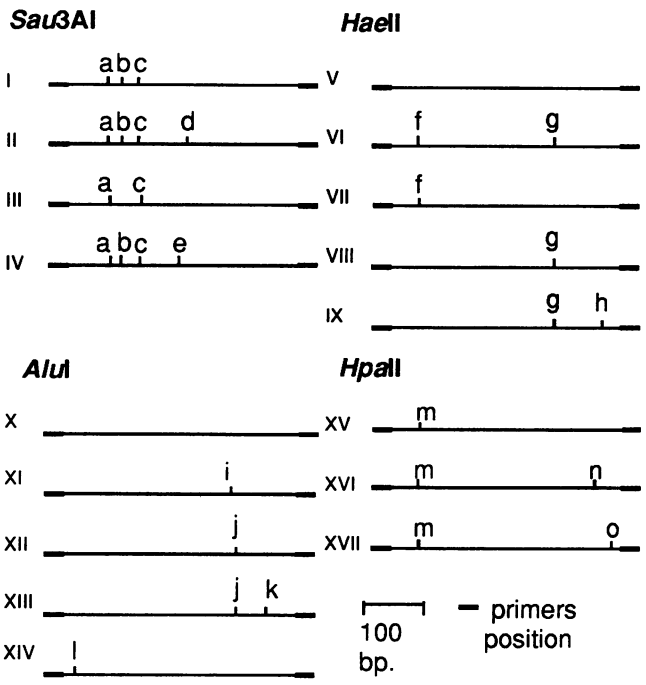

FIG. 2. Restriction site map corresponding to RFLP profiles of *Sau*3AI, *Hae*II, *Alu*I, and *Hpa*II. Each map is represented only between the two primers in the amplified *pelY* gene region. Roman numerals indicate names of profiles and are used to define the RFLP groups (Table 1).

carotovora subsp. *carotovora* strains. A further subdivision grouped 11 *E. carotovora* subsp. *odorifera* strains among the 12 tested (Fig. 3, RFLP groups 3, 4, 16, 5, and 7). The C cluster grouped the atypical *E. carotovora* subsp. *atroseptica* strains (growing at 37°C) and most of the indole-producing *E. carotovora* subsp. *carotovora* strains. RFLP groups 21 (*E. carotovora* subsp. *wasabiae* strains) and 19 (two atypical *E. carotovora* subsp. *carotovora* strains from Cuba) constituted two distant

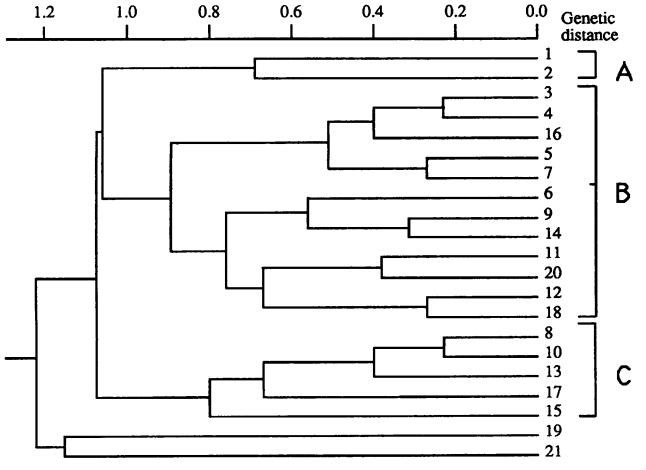

FIG. 3. Phenogram obtained by unweighted average pair-group method of clustering classification based on the whole RFLP data set. Numbers indicate RFLP groups (see Table 1), and A, B, and C represent the three main domains of the tree.

and separated clusters. This phenetic analysis grouped the *atroseptica* and *wasabiae* subspecies into two distant clusters.

With the other (cladistic) analysis methods, RFLP groups 21 and 19 were placed in A and B domains, respectively, and appeared very distant from each other. The two cladistic methods ("restml" and "mix" programs) clustered groups 1, 2 (*E. carotovora* subsp. *atroseptica*), and 21 (*E. carotovora* subsp. *wasabiae*) into the same stem of the trees (data not shown). Because the location of the RFLP groups 5, 6, 7, and 16 was variable with cladistic analyses, grouping of *E. carotovora* subsp. *odorifera* strains appeared less reliable (data not shown). However, on the basis of the *Sau*3AI digestion patterns these three domains were conserved with a cladistic analysis method ("restml" program, data not shown), and diversification among the main groups was revealed by addition of the other restriction enzyme data.

DISCUSSION

Our purpose, at the beginning of this study, was to develop one identification tool for typical blackleg agent in Europe (*E. carotovora* subsp. *atroseptica* isolated from potato) and another tool with a broader specificity to detect all *E. carotovora* strains. PCR and RFLP applied to *pel* genes provided an answer to this problem, and these results could be further developed for diagnosis purposes.

In this study, all the *E. carotovora* strains tested (89 strains) were detected except *E. carotovora* subsp. *betavasculorum* (4 strains). This lack of detection might be correlated with the host range of these bacteria: indeed, sugar beet pectins are highly acetylated (24 to 50%) compared with most of the plants (31). We hypothesize that the enzyme in *E. carotovora* subsp. *betavasculorum* and, consequently, the sequence of its gene are different.

Our PCR test was developed with sequences limited to the open reading frames of a *pel* gene which is shown by this work to be present in *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *odorifera*, and *E. carotovora* subsp. *wasabiae*. *pel* genes are thought to play a role in pathogenicity (18, 30). Although it has been shown that the inactivation of the *pel153* gene does not reduce the maceration ability of the bacterium on potato tuber (38), the ubiquity of the *pelY* gene found in *E. carotovora* might reveal the involvement of this gene in other steps, e.g., a systemic phasis, of phytopathogenicity.

Identification methods for pathogenic bacteria using genes involved in pathogenicity have been developed already (9). However, because they are not neutrally selected, it is possible that the use of genes involved in pathogenicity might introduce a bias in phenetic and phylogenic studies. Our studies of the *pelY* family were performed essentially for comparisons with those previously established with data sets more representative of the whole genome (12, 29). For this reason, most of the strains we used were identical to those studied by Priou (29).

RFLP analysis showed that *E. carotovora* subsp. *atroseptica* strains constitute a homogeneous group, whatever the original country and the year of isolation; this could reflect a stable, host-specialized population. This conclusion is in accordance with other studies (4, 12, 29). However, subgrouping was observed as shown with phenotypic and genotypic data (29, 33); the significance of this subgrouping is unknown. The *Sau*3AI digestion profile (profile IV, Fig. 2) clearly identified typical *E. carotovora* subsp. *atroseptica* strains giving typical blackleg symptoms on potato (29). Interestingly, two strains of *E. carotovora* subsp. *atroseptica* isolated from tomato showed the same profile. These strains caused typical blackleg symp-

toms on tomato and on potato (1, 29). This suggests that these strains (both in RFLP group 2) were potato pathogens that can occur on tomato, which has a genome very similar to that of potato (2).

The exact relationship between *E. carotovora* subsp. *atroseptica* and subsp. *wasabiae* remains to be solved. These two kinds of bacteria, pathogenic in cold climates (13, 28), were found to be clearly distant by phenetic analysis (Fig. 3) but to be closely related by cladistic analyses (data not shown).

Even though *E. carotovora* subsp. *odorifera* strains were previously determined to be atypical *E. carotovora* subsp. *atroseptica*, no clear distinction has been observed with our whole data set between *E. carotovora* subsp. *odorifera* and *E. carotovora* subsp. *carotovora* strains. Other authors were able to differentiate these two subspecies either as closely (12, 29) or as more distantly (7, 39) related groups.

A great genetic diversity was observed among strains of *E. carotovora* subsp. *carotovora*. Two main clusters appeared, the first one grouping the indole-producing strains with the strains previously identified as atypical *E. carotovora* subsp. *atroseptica* (exhibiting pathogenicity traits of *E. carotovora* subsp. *carotovora* [29]) and the second one closely related to *E. carotovora* subsp. *odorifera* strains. This diversity may result from a nonmonophyletic origin and agrees with the broad host range and geographical distribution of this subspecies.

Two strains (88.29a1 and CIP009) were not clearly defined by other studies, because they showed a mixture of *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* phenotypic and genotypic characteristics and exhibited pathogenicity traits of *E. carotovora* subsp. *atroseptica* (29). In our study, these strains appeared in *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera* RFLP groups (Table 1).

In conclusion, our PCR-RFLP test, linked to pathogenicity studies, could be used to monitor the diversity of the *E. carotovora* populations, provided that this test is applied to a larger number of strains.

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