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Characterisation of *Erwinia carotovora* subspecies and detection of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR-based methods

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Abstract

A PCR-RFLP test based on a pectate-lyase encoding gene permits the detection of several *Erwinia carotovora* subspecies, but requires complete DNA extraction. This paper reports on the suitability of a simplified PCR-RFLP protocol to characterise *E. carotovora* strains and on the performance of PCR, using the same primers, to detect the *atroseptica* subspecies in substrates of epidemiological significance. A collection of 140 strains from various hosts and geographical origins was characterised for biochemical traits and PCR-RFLPs. PCR performed on boiled bacterial suspensions yielded an amplification product of 434 bp in 109 of the 140 strains. None of the *E. carotovora* subsp. *betavasculorum* strains was amplified, even after complete DNA extraction. RFLPs of the PCR product yielded 24 groups, 3 of which were new. Twenty one groups were specific to one subspecies. Several strains biochemically similar to *E. carotovora* subsp. *atroseptica*, but growing at 37 °C, showed PCR-RFLP profiles characteristic of *E. carotovora* subsp. *carotovora*. Phenetic and cladistic analyses gave three main domains, not strictly related to hosts or geographical origins. The *atroseptica* (RFLP groups 1 and 2) and *wasabiae* (group 21) subspecies constituted one of the domains, despite clustering distantly from one another. Host specialisation and molecular homogeneity suggest a clonal structure within these subspecies. Conversely, *E. carotovora* subsp. *odorifera*, despite its limited host range and geographical distribution, and *E. carotovora* subsp. *carotovora* showed great molecular diversity, spreading respectively across five and 19 RFLP groups. These two subspecies shared RFLP groups 4, 5 and 6. The tree nodes in the phenograms showed a low robustness when bootstrapping the data matrix. PCR coupled with a 48h enrichment step in a polypectate-rich medium improved detection thresholds of *E. carotovora* subsp. *atroseptica* ($1.5.10^2$- $1.5.10^3$ bacteria/ml in leaves, stems, and tuber peel extracts to 4.10^7 bacteria/ml in wash water) relative to either immunomagnetic separation coupled with PCR or DAS-ELISA (2.10^5 in plant samples to 2.10^7 bacteria/ml in wash water).

Introduction

Under temperate conditions, soft rot of potato crops is primarily caused by two of the five subspecies recognised within *E. carotovora*. While *E. carotovora* subsp. c*arotovora* is widespread and has a wide host range besides potato, *E. carotovora* subsp. *atroseptica* is more specifically associated with potato and is prevalent under cold to temperate climates. Both subspecies are responsible for severe losses in the field and during storage. The other three subspecies of *E. carotovora* have been more recently described.

E. carotovora subsp. *betavasculorum* causes soft rot of sugar beet (Thomson et al., 1981), and was also recovered from sunflower, artichoke and potato (Samson et al., 1998). *E. carotovora* subsp. *wasabiae* (Goto and Matsumoto, 1987) was isolated from Japanese horseradish and *E. carotovora* subsp. *odorifera*, previously designated as atypical *E. carotovora* subsp. *atroseptica* (Samson et al., 1980), was isolated from witloof-chicory, leek, allium, and celery (Gallois et al., 1992). Although these two subspecies have not been isolated from potato so far, their significance for this crop cannot be ruled out because of diagnostic difficulties and the possible co-infection of a single host by several subspecies. Therefore specific and sensitive methods for identifying and detecting the various *E. carotovora* subspecies are required to improve diagnosis and to investigate the epidemiology of potato soft rot.

Methods based on biochemical tests on isolated bacteria, serology, and more recently, molecular biology, have been developed to detect and characterise *Erwinia carotovora*. However, not all of them can be used routinely for analyses on potato tubers, or allow specific detection of each *E. carotovora* subspecies. Biochemical tests (Lelliot and Dickey, 1984) permit the differentiation of all subspecies, but can only be applied to purified bacterial cultures. The crystal violet pectate medium (Cuppels and Kelman, 1974), selective for pectinolytic Erwinias, avoids the purification stage, but does not discriminate between *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *odorifera* and *E. carotovora* subsp. *wasabiae*. Furthermore, both methods are time-consuming and are not sensitive enough for testing purposes. Serological techniques do not require isolation of the bacteria, but their efficiency depends on the specificity of the antibodies. Monoclonal antibodies are more specific than polyclonal antisera (Gorris et al., 1994), but have a lower affinity (De Boer and MacNaughton, 1987). Serological techniques are not sensitive enough to detect low, but epidemiologically significant, bacterial populations. To improve the detection thresholds, a PCR test based on amplification of the pectate lyase-encoding gene (*pel* gene) was developed, and allows identification of all *E. carotovora* strains except *E. carotovora* subsp. *betavasculorum* (Darrasse et al., 1994). The analysis of restriction fragment length polymorphisms (RFLP) of the amplified product differentiates *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *wasabiae*, which belong to specific RFLP groups, while *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera* strains could not be clearly distinguished. More recently, primers were selected for detection of *E. carotovora* subsp. *atroseptica* using PCR only (De Boer and Ward, 1995; Fréchon et al., 1995); no such specific primers are available to date for the other subspecies.

The objectives of this work were to assess the applicability of a simplified PCR-RFLP protocol for the identification of *E. carotovora* subspecies, and to evaluate the performance of PCR for detection of *E. carotovora* subsp. *atroseptica* in various substrates of potential epidemiological significance. To this end, an extended collection of strains, representing a large diversity of hosts, dates and places of origin, was characterised using both standard biochemical tests and PCR-RFLP, in order i) to test the specificity of molecular technology for the diagnosis of *E. carotovora* subspecies and ii) to investigate possible relationships between molecular diversity and geographical or host origins. Furthermore, the detection thresholds of *E. carotovora* subsp. *atroseptica* introduced into plant and soil extracts or wash water were tested for the PCR technique and compared with DAS-ELISA and immunocapture-PCR using a monoclonal antibody. The data show that PCR-RFLP can be used to identify *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *wasabiae*, and most strains of *E. carotovora* subsp. *odorifera* and *E. carotovora* subsp. *carotovora*, and that PCR using the same set of primers provides a valuable tool for detecting *E. carotovora* subsp. *atroseptica* in the different substrates investigated.

Materials and methods

Bacterial cultures and inoculum preparation

Thirty-two strains of *E. carotovora* subsp. *atroseptica*, 92 strains of *E. carotovora* subsp. *carotovora*, 5 strains of *E. carotovora* subsp. *betavasculorum*, 6 strains of *E. carotovora* subsp. *odorifera*, and 5 strains of *E. carotovora* subsp. *wasabiae* (Table 1) were used. Bacteria were stored on yeast extract-peptone agar slants (0.3% yeast extract, 0.5% peptone, 1.5% agar) at room temperature, and grown on King's medium B at 27 °C before use.

For the detection studies, bacterial suspensions in distilled water were prepared from a 24 h-old culture grown at 27 °C on King's B medium of one *Erwinia carotovora* subsp. *atroseptica* strain (*Eca* 86.20), and the concentration was adjusted to 2–4 10^9

Table 1. Origins and PCR-RFLP groups of *E. carotovora* strains classified according to their biochemical and physiological traits

Strains	Isolated from	Geographic origin and year of isolation	RFLP group
Erwinia carotovora subsp.*atroseptica*			
86.20*	Potato	France, 1986[a]	1
87.7*	Potato	France, 1987[a]	2
EII 216	Potato	France, 1975[b]	1
EII 217	Potato	France, 1975[b]	1
EII 219	Potato	France, 1975[b]	1
EII 221	Potato	France, 1975[b]	1
EII 224	Potato	France, 1975[b]	1
EII 226	Potato	France, 1975[b]	1
EII 230	Potato	France, 1976[b]	1
EII 234	Potato	France, 1976[b]	2
EII 316	Potato	USA, 1977[b]	1
EII 322	Potato	USA, 1977[b]	1
EII 326	Potato	USA, 1977[b]	2
EII 328	Potato	USA, 1977[b]	1
EII 331	Potato	France, 1977[b]	1
EII 332	Potato	Germany, 1977[b]	1
EII 336	Potato	France, 1977[b]	1
EII 339	Potato	France, 1977[b]	1
EII 349	Potato	Spain, 1977[b]	2
EII 351	Potato	USA, 1976[b]	1
EII 360	Potato	USA, 1977[b]	2
EII 388	Potato	France, 1978[b]	1
EII 412	Potato	France, 1978[b]	1
EII 451	Potato	France, 1979[b]	1
EII 458	Potato	France, 1979[b]	1
S19-4	Potato	Tunisia, 1994[c]	1
5d	Potato	Argentina[d]	1
22d	Potato	Argentina[d]	2
Erwinia carotovora subsp. *carotovora*			
87.25*	Potato	France, 1987[a]	10
91.8	Potato	France, 1991[a]	4
93.13	Potato	Congo, 1993[a]	24
94.1	Potato	Marocco, 1994[a]	8
EII 135[e]	Tomato	France, 1974[b]	8
EII 136	Iris	France, 1974[b]	9
EII 167	Cyclamen	France,1974[b]	9
EII 183	Cyclamen	France, 1975[b]	12
EII 184	Iris	France, 1975[b]	9
EII 205	Lettuce	France, 1975[b]	4
EII 210	Potato	France, 1975[b]	14
EII 220	Potato	France, 1975[b]	14
EII 222	Potato	France, 1975[b]	9
EII 223	Potato	France, 1975[b]	9
EII 252	Stream water	France, 1974[b]	14
EII 254	Stream water	France, 1974[b]	14
EII 259	Stream water	France, 1974[b]	14
EII 261	Stream water	France, 1974[b]	14
EII 278	Celery	France, 1976[b]	4
EII 290	Potato	USA, 1976[b]	9
EII 291	Potato	USA, 1976[b]	6
EII 311	Philodendron	France, 1977[b]	24
EII 319	Potato	USA, 1977[b]	6
EII 320	Potato	USA, 1976[b]	6
EII 324	Potato	USA, 1976[b]	13
EII 327	Cauliflower	France, 1976[b]	16
EII 330	Potato	France, 1977[b]	18
EII 345	Tomato	France, 1977[b]	9
EII 352[e]	Potato	USA, 1976[b]	8
EII 356	Tomato	Algeria[b]	8
EII 362	Sunflower	France, 1977[b]	9
EII 364	Sunflower	France, 1977[b]	11
EII 368	Arum	France, 1977[b]	24
EII 372	Tomato	France, 1977[b]	24
EII 393	Lettuce	France, 1978[b]	14
EII 394	Sunflower	France, 1978[b]	9
EII 406	Potato	France, 1978[b]	9
EII 407	Potato	France, 1978[b]	9
EII 409	Potato	France, 1978[b]	4
EII 411	Potato	France, 1978[b]	14
EII 413	Primula	France, 1978[b]	9
CFBP 21.36	Potato	France, 1976[f]	9
CFBP 21.37	Potato	France, 1976[f]	9
CFBP 21.40	Potato	France, 1976[f]	4
CFBP 21.41	Potato	France, 1978[f]	9
AS24-2	Potato	Tunisia, 1993[c]	23
AS103-3	Potato	Tunisia, 1994[c]	14
M7-1	Potato	Tunisia, 1994[c]	8
M3-2	Potato	Tunisia, 1994[c]	8
B2-5	Potato	Tunisia, 1994[c]	4
L1-4	Potato	Tunisia, 1994[c]	6
S18-2	Potato	Tunisia, 1994[c]	8
S8-1	Potato	Tunisia, 1994[c]	8
S11-5	Potato	Tunisia, 1994[c]	8
S23-1	Potato	Tunisia, 1994[c]	6
48b	Potato	Argentina[d]	8
42c	Potato	Argentina[d]	14
52c	Potato	Argentina[d]	6
25d	Potato	Argentina[d]	9
29d	Potato	Argentina[d]	9

Table 1. Continued

Strains	Isolated from	Geographic origin and year of isolation	RFLP group
31d	Potato	Argentina[d]	9
38ϵ	Potato	Argentina[d]	6
17f	Potato	Argentina[d]	8
5	Potato	Argentina[d]	6
12a	Potato	Argentina[d]	9
26b	Potato	Argentina[d]	22
29a	Potato	Argentina[d]	8
EII 223	Potato	France, 1975[b]	9
EII 252	Stream water	France, 1974[b]	14
123a	Potato	Argentina[d]	9
77a	Potato	Argentina[d]	4
89a	Potato	Argentina[d]	6
67c[e]	Potato	Argentina[d]	8
17b	Potato	Argentina[d]	6
35b	Potato	Argentina[d]	6
39b	Potato	Argentina[d]	6
90a	Potato	Argentina[d]	6
131b	Potato	Argentina[d]	6
28b	Potato	Argentina[d]	9
31a	Potato	Argentina[d]	9
37b	Potato	Argentina[d]	9
79a	Potato	Argentina[d]	9
86a	Potato	Argentina[d]	9
112a	Potato	Argentina[d]	9
115a	Potato	Argentina[d]	9
118a	Potato	Argentina[d]	14
129a	Potato	Argentina[d]	9
4a	Potato	Argentina[d]	6
44b	Potato	Argentina[d]	6
100a	Potato	Argentina[d]	6
135b	Potato	Argentina[d]	6
Atypical strains			
EII 338 [e,g]	Potato	France, 1977[b]	9
EII 354 [e,g]	Pepper	Algeria, 1977[b]	8
22ϵ[g]	Potato	Argentina[d]	9
78a[h]	Potato	Argentina[d]	8
7b[h]	Potato	Argentina[d]	8
65a[h]	Potato	Argentina[d]	8
2a[h]	Potato	Argentina[d]	8
Erwinia carotovora subsp. *betavasculorum*			
CFBP 1520*	Sunflower	Mexico[f]	Not amplified
SF 142.2*	Artichoke	La Réunion, 1986[b]	Not amplified
CFBP 21.22*	Sugar beet	USA, 1972[f]	Not amplified
CFBP 3292	Sugar beet	USA, 1974[f]	Not amplified
CFBP 3294	Sugar beet	USA[f]	Not amplified
Erwinia carotovora subsp. *odorifera*			
EII 201	Celery	France, 1975[b]	5
CFBP 3259	Leek	France, 1980[f]	3
CFBP 3260	Leek	France, 1980[f]	3
CFBP 3261	Leek	France, 1982[f]	6
CFBP 3296	Hyacinth	The Netherlands[f]	3
CFBP 1878*	Witloof chicory	France, 1978[f]	4
Erwinia carotovora subsp. *wasabiae*			
CFBP 3304*	Horseradish	Japan[f]	21
CFBP 3305	Horseradish	Japan[f]	21
CFBP 3306	Horseradish	Japan[f]	21
CFBP 3307	Horseradish	Japan[f]	21
CFBP 3308*	Horseradish	Japan[f]	21

[a] Bernard Jouan, Institut National de la Recherche Agronomique, Rennes, France, personal collection.
[b] Régine Samson, Institut National de la Recherche Agronomique, Angers, France, personal collection.
[c] Sylvie Priou, Centre International de la Pomme de terre, Tunisia, personal collection.
[d] Sylvia Capezio, Instituto Nacional de Tecnologia Agropecuaria, Argentina, personal collection.
[e] indole production from tryptophan.
[f] CFBP: Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France.
[g] Strains with biochemical features of *E. carotovora* subsp. *carotovora* but unable to grow at 37 °C.
[h] Strains with biochemical features of *E. carotovora* subsp. *atroseptica* but able to grow at 37 °C.
* Reference strains included as controls for RFLP profiles.

bacteria.ml^{-1} by spectrophotometry (optical density at 350 nm). Ten-fold dilutions of the bacterial suspensions, from 2–4 10^9 to 2–4 bacteria.ml^{-1}, were prepared and used to inoculate various substrates.

Biochemical tests

All strains were reisolated on King's B medium before being tested for selected differential biochemical features according to Thomson et al. (1981), Lelliot and Dickey (1984), Goto and Matsumoto (1987), and Gallois et al. (1992) (Table 2). *E. carotovora* subsp. *atroseptica* strains and *E. carotovora* subsp. *carotovora* strains were examined for calcium polygalacturonate liquefaction in Sutton's medium, tested as modified by Bonnet (1973), to assess the pectinolytic capacity of the strain, and for lactose acidification and malonate alkalinisation in (A) medium [($NH_4H_2PO_4$,

Table 2. Biochemical and physiological properties used for the identification of *Erwinia carotovora* subspecies. + and − indicate the presence of the trait in more than 90% and in less than 10% of the strains, respectively

Nutritional and physiological features [a]	*E. carotovora* subspecies [b]				
	Eca	*Ecc*	*Eco*	*Ecb*	*Ecw*
Acidification of:					
Lactose	+	+	+	+	+
α-Methylglucoside	+	-	+	+	-
Melibiose	+	+	+	-	-
Sorbitol	-	-	+	-	-
D-Arabitol	+	-	+	-	-
Inulin	-	-	-	+	-
Indole production	-	+/-[c]	-	-	-
Citrate alkalinisation	+	+	+	-	+
Growth at 37 °C	-	+	+	+	-
Production of reducing substances from sucrose	+	-	+	+	-

[a] Data from Lelliot and Dickey (1984), Thomson et al. (1981), Goto and Matsumoto (1987), Gallois et al. (1992).
[b] *Eca*, *E. carotovora* subsp. *atroseptica*; *Ecc*, *E. carotovora* subsp. *carotovora*; *Eco*, *E. carotovora* subsp *odorifera*; *Ecb*, *E. carotovora* subsp. *betavasculorum*; *Ecw*, *E. carotovora* subsp. *wasabiae*.
[c] +/-, 11 - 89% positive strains.

0.1%; KCl, 0.02%; $mgSO_4.7H_2O$, 0.02%; bromothymol blue, 0.008%; carbohydrate, 0.3% w/v, pH 6.8)]. This medium, derived from Ayers' basal medium (Ayers et al., 1919), was also used to determine the indole production (Bonnet, 1973). These two tests differentiate *E. carotovora* from *E. chrysanthemi.* Methods described by Dye (1968) were used to test growth at 37 °C and utilisation of α-methylglucoside. The production of reducing substances from sucrose was studied (Dye, 1968) for *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* strains atypical by their growth at 37 °C.

The bacteria received as *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *betavasculorum* and *E. carotovora* subsp. *wasabiae* were submitted to complementary biochemical tests. Carbon sources were tested by acidification (melibiose, D-arabitol, sorbitol) or alkalinisation of (A) liquid medium mixed with 0.5% (w/v) of carbon sources filter sterilised through 0.2 μm millipore. Inulin assimilation was studied in a peptone medium [$(NH_4)_2SO_4$ 0.2%, yeast extract 0.05%, tryptone 0.1%, inulin 0.5%, red phenol 0.02%, pH 7.5)].

PCR-RFLP protocols

Bacterial strains were grown overnight on King's B at 26 °C; a few bacterial colonies were picked and mixed thoroughly in 500 μl sterile, ultrapure water. After boiling for 10 min in a water bath to lyse bacteria, 10 μl of the resulting suspension were directly used for PCR. When DNA was not amplified, lysis of bacteria was improved by adding 0.5% Tween 20 to the bacterial suspension before boiling. When adding this detergent was not sufficient, total DNA was extracted before being amplified as previously described (Darrasse et al., 1994).

PCR was performed in a 9600 Perkin-Elmer Cetus thermal cycler using primers Y1 (5′-TTACCGGACGCCGAGCTGTGGCGT-3′) and Y2 (5′-CAGGAAGATGTCGTTATCGCGAGT-3′), specific for *E. carotovora* and selected from the pectate lyase encoding gene (Darrasse et al., 1994). Amplification was carried out in a 100 μl reaction mixture, containing 10 μl of the amplification buffer supplied with the enzyme, 10 nmol each of the deoxynucleotides dATP, dCTP, dGTP, and dTTP, 50 pmol of each primer, and 2 units of *Taq* DNA polymerase (Appligene, Illkirch, France). Amplification conditions were modified as follows: DNA was denatured at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and polymerisation at 72 °C for 45 s. Aliquots of PCR products (10 μl) were analysed by electrophoresis through 1.5% agarose (Appligene, Illkirch, France) minigels containing 0.5 μg/ml ethidium bromide. A standard 1 Kb ladder (GIBCO/BRL, Gaithersburg, USA) was included on each gel.

Amplified DNA remaining after assessing amplification (90 μl) was precipitated by adding 1/10 sodium acetate (3M) and 2.5 volumes of cold (−20 °C) absolute ethanol, and pelleted by centrifugation at 12,000 g during 30 min. Pellets were dried 1 h at 36 °C and resuspended in 50 μl of Tris-EDTA buffer (Maniatis et al., 1982). Aliquots (5 μl) of concentrated DNA were digested at 37 °C during 2-4 h with *Alu*I, *Hae*II, *Hpa*II, and *Sau*3AI (Boehringer GmbH, Mannheim, Germany), according to the manufacturer's recommendations, in a 20 μl final volume. The digestions were terminated by the addition of 4 μl gel tracking dye (Maniatis et al., 1982). Samples (12 μl) were separated by electrophoresis through 2.7% high resolution agarose gels (Appligene, Illkirch, France, Sigma, St Louis, USA). A standard DNA marker V (Boehringer) was included on each gel, before staining with 10 μg/ml of ethidium bromide for 10-15 min.

Fragment sizes were determined by comparison with the size marker, and RFLP groups were determined by examining the combined digestion results with the four enzymes (Darrasse et al., 1994). New RFLP patterns were confirmed by electrophoresis through a high resolution, 9% polyacrylamide gels.

Phenetic and phylogenic data analyses

In order to encompass the total diversity described so far in *E. carotovora*, phenetic and cladistic studies were applied to the data set including our results and those of Darrasse et al. (1994).

Phenetic analysis

Each restriction site was scored as 1 (present) and 0 (absent) for all four enzymes. The pair-wise distance matrix, calculated as the complements to one of the Sorensen-Dice similarity coefficient (Jackson et al., 1989), was subjected to cluster analysis using the unweighted average pair-group method (UPGMA) option in the Neighbor module of the Phylogenetic Inference Package (PHYLIP 3.5) developed by Felsenstein (1993).

Cladistic analyses

The 0-1 matrix was used as input file for two cladistic analyses by programs (Mix and Restml) of the PHYLIP 3.5 software. The Mix module generates cladograms based on parsimony analysis for any kind of marker sets, whereas Restml was specifically designed for processing restriction polymorphism data using a maximum likelihood computing algorithm. The Mix program was run ten times with random input orders of the strains, with the options U and P= Wagner parsimony. The majority rule consensus tree, generated by the Consense module of PHYLIP 3.5 from the output trees of the ten runs, was visualised with the TREEVIEW software (Page, 1996). The Restml module was also run ten times with the options U, L=4 and E=100.0, and the best tree, chosen as the one with the highest log-likelihood value, was drawn with the TREEVIEW software.

Bootstraps

The stability of the phenetic and cladistic results were tested by resampling the input data set. Bootstraps were applied to the original 0-1 matrix using the Seqboot module of PHYLIP 3.5 before performing phenetic and cladistic studies. Consensus trees were obtained with the Consense module and drawn using the TREEVIEW program.

Preparation of plant, soil and water substrates

Detection tests were applied to different potato organs (leaves, stems, tuber peels) and substrates (wash water, soil). Stems and leaves were sampled from plants (cv Bintje) cultivated in the greenhouse at 20 °C for 1–2 months. Periderm was removed with a hand-peeler from certified (class A) seed tubers, stored at 4 °C and washed in distilled water. The wash water was kept for detection tests. All plant samples (leaves, stems and peels) were crushed in a custom bead-press before use. A soil mixture in equal proportions of sand, loam, and peat was heat treated at 100 °C for one h before inoculation.

For each bacterial concentration, one g of plant material (leaves, stem or potato peel) was crushed in 5 ml of SCPAP buffer (0.1% trisodium succinate, 0.1% trisodium citrate, 0.15% K_2HPO_4, 0.1% KH_2PO_4, 2% polyvinylpolypyrrolidone, 0.02 m ascorbic acid) (Minsavage et al., 1994) and 1 ml of bacterial suspension. After the liquid phase was collected, each crushed sample was rinsed with 20 ml of SCPAP buffer, which was recovered and added to the liquid already obtained. Each extract was then submitted to DNA extraction, either immediately or after immunomagnetic separation or enrichment in a selective medium. For detection by DAS-ELISA, a similar protocol, but with SCPAP replaced with PBS buffer (0.8% NaCl; 0.02% KH_2PO_4; 0.29% Na_2PO_4, $12H_2O$; 0.02% KCl; pH 7.2) supplemented with 0.2% sodium diethyldithiocarbamate (DIECA), was used.

One hundred ml of distilled water in which tubers had been washed were artificially contaminated with 1 ml of bacterial suspension, before being centrifuged at 1000 g for 10 min. Supernatants were transferred to clean tubes, and centrifuged at 1000 g for another 10 min after adding 2% of polyvinylpolypyrrolidone. Supernatants were recovered and centrifuged at 10,000 g for 20 min. DNA was extracted immediately from pellets resuspended in 10 ml of TE 50 X (Tris 500 mm, EDTA 50 mm, pH 8.0), or after either enrichment or immunocapture from pellets resuspended in a RTB solution, consisting of 10 ml Ringer's solution (0.225% NaCl, 0.0105% KCl, 0.006% $CaCl_2$, $2H_2O$, 0.005% $NaHCO_3$) supplemented with 0.1% Tween 20 and

0.1% bovin serum albumin. The PBS-DIECA buffer (10 ml) was used to resuspend centrifuged pellets submitted to DAS-ELISA.

Soil samples (1 g), homogenised in 100 ml of distilled water, were artificially contaminated with one ml of the various bacterial suspensions before being submitted to the protocol used for wash water.

Enrichment step

Enrichment was performed on preparations from the various samples by mixing 500 μl of each extract with an equal volume of the D-PEM medium (Gorris et al., 1994), selective for pectinolytic *Erwinia* species, and incubating for 48 h at 27 °C. After enrichment, samples were centrifuged for 10 min at 12,000 g before DNA extraction.

Immunomagnetic separation (IMS)

Immunomagnetic separation was performed on all substrates following the same protocol, derived from Van der Wolf et al. (1996). Five hundred μl of a 1/2000 dilution in RTB of the monoclonal antibody 4G4 (Gorris et al., 1994) were added to 500 μl of each extract, distributed in Eppendorf tubes. The monoclonal antibody 4G4 was preferred to polyclonal antibodies because of its higher specificity to *E. carotovora* subsp. *atroseptica* (Gorris et al., 1994), despite a lower binding capacity (Van der Wolf et al., 1996). After shaking gently for 20 min at room temperature in a magnetic concentrator (Dynal MPC-M, Oslo, Norway), paramagnetic particles conjugated with goat anti-mouse antibodies (Advanced Magnetics, Cambridge, MA USA, 8-4340) were added at a final concentration of 50 μg/ml. Tubes were incubated for a further 20 min at room temperature with gentle shaking. Separation was achieved by applying the supplied strip magnet against the tubes for 2 min. Supernatants were carefully removed with a micropipette. The pellets were rinsed in 1 ml RTB, and again magnetically separated from the buffer. Pellets were finally suspended in 500 μl RTB, and centrifuged at 12,000 g for 10 min before DNA extraction.

DNA extraction and PCR amplification

DNA extraction from plant extracts

Two hundred μl of each extract were incubated with 400 μl of Tris Borate EDTA, pH 8.0 (TBE) lysis buffer (0.75% N-laurylsarcosine, 0.015% proteinase K, 0.005% RNAse A, 1% lysozyme) at 37 °C for 90 min. One hundred μl of 5M NaCl and 80 μl of CTAB-NaCl solution (4.1% NaCl and 10% CTAB) were added. After incubation at 65 °C for 10 min, samples were extracted with 780 μl of chloroform-isoamyl alcohol (24:1). Extracted DNA was precipitated by addition of 0.6 volume of isopropanol and tubes were placed at −20 °C for 1 h. After centrifugation at 12,000g for 20 min, the pellets were washed in 1 ml of 70% ethanol and centrifuged again at 12,000g for 20 min. Pellets were dried at 37 °C before being suspended in 50 μl of TE 1X and kept at −20 °C until further use.

DNA extraction from wash water and soil extracts

DNA extraction was performed on pellets obtained by centrifuging 500 μl of extracts for 10 min at 12000 g. Pellets were suspended in 100 μl of a lysis solution (0.015% proteinase K, 0.15% lysozyme, 0.015% RNaseA, 0.2% DIECA) prepared in 1X PCR buffer (Appligene, Illkirch, France), incubated at 37 °C for 2–3 h, and heated to 90 °C for 10 min before being extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). Extracted DNA was precipitated by adding 1/10 volume of 3M sodium acetate and 2 volumes of absolute ethanol, and incubating at −20 °C for 1 h. The pellets obtained by centrifuging for 20 min at 12,000 g were washed in 1 ml 70% ethanol, and centrifuged for a further 30 min at 12,000 g. Pellets were dried at 37 °C before being suspended in 50 μl of TE 1X and kept at −20 °C before use in the PCR reaction. The same procedure was applied to soil extracts and wash water.

PCR reactions

Amplification conditions for the PCR reaction were identical to those used in the PCR-RFLP protocol previously described, except that PCR was carried out on 5 μl of the DNA extracts in a total of 50 μl reaction mixture. DNA was denatured by boiling for 5 min before being added to the reaction mix. Am-

plification products were analysed by electrophoresis through 1.5% agarose gels as described above.

Das-Elisa

Bacterial detection by double-antibody-sandwich-ELISA (DAS-ELISA) with the monoclonal antibody 4G4 was performed with the commercial kit REALISA^{TM} (DURVIZ C.E., Valencia, Spain) on plant, soil and wash water extracts with or without previous enrichment. The kit was used according to the manufacturer's instructions, except that 100 μl of the original extract was used per well instead of 50 μl.

Results

Biochemical results

Biochemical tests permitted the identification of all *E. carotovora* subspecies. The results were generally in accordance with the determinations provided by the different authors who sent the strains. However, seven strains (AS24-2, AS103-3, M3-2, B2-5, L1-4, S18-2, S23-1), originally described as atypical *E. carotovora* subsp. *carotovora* because they did not grow at 37 °C, were typical for this trait, growing at this temperature in our culture conditions. Strains received as *E. carotovora* subsp. *carotovora* (EII 201), *E. carotovora* subsp. *atroseptica* (EII 352, 12a, 26b) and *E. chrysanthemi* (67c) were identified by our tests as *E. carotovora* subsp. *odorifera* (EII 201) and *E. carotovora* subsp. *carotovora* (EII 352, 12a, 26b, 67c). The ability to grow at 37 °C was in accordance with the determination provided by biochemical tests (Table 2), except for four *E. carotovora* subsp. *atroseptica* and three *E. carotovora* subsp. *carotovora* respectively able and unable to grow at this temperature. Moreover, five *E. carotovora* subsp. *carotovora* strains produced indole from tryptophan (EII 135, EII 352, EII 338, EII 354, 67c); two of these were atypical for growth at 37 °C (EII 338, EII 354).

PCR results

All products obtained after PCR amplification were of the expected size (434 bp). All 32 *E. carotovora* subsp. *atroseptica* strains tested, including atypical strains, showed an amplification product after boiling; however, 18 of the *E. carotovora* subsp. *carotovora* (EII 135, EII 167, EII 184, EII 222, EII 278, EII 291, EII 311, EII 327, CFBP 21.41, M3.2, 5, 17b, 35b, 39b, 4a, 44b, 135b, S23.1) and two of the six *E. carotovora* subsp. *odorifera* (CFBP 3260, CFBP 3261) tested failed to produce an amplification product, as did the five *E. carotovora* subsp. *wasabiae*, using this rapid and simple procedure. Addition of Tween 20 as a detergent improved lysis, allowing amplification of eight more strains of *E. carotovora* subsp. *carotovora* (M3.2, 5, 17b, 35b, 39b, 4a, 135b, S23.1). However, extraction of DNA was necessary to obtain an amplification product with the remaining ten *E. carotovora* subsp. *carotovora*, two *E. carotovora* subsp. *odorifera* and five *E. carotovora* subsp *wasabiae* strains. DNA extracted from each of these strains was successfully amplified, except for the *E. carotovora* subsp. *betavasculorum* strains, confirming previous results (Darrasse et al., 1994).

RFLP analysis

The 135 *Erwinia carotovora* strains tested by RFLP were distributed in 16 of the 21 RFLP groups previously described (Darrasse et al., 1994). All *E. carotovora* subsp. *atroseptica* strains belonged to RFLP groups 1 and 2, specific of this subspecies. Four *E. carotovora* subsp. *atroseptica* growing at 37 °C (78a, 7b, 65a, 2a) were grouped in RFLP group 8, together with one (EII 354) of the three *E. carotovora* subsp. *carotovora* not growing at 37 °C and with 11 other *E. carotovora* subsp. *carotovora*. *E. carotovora* subsp. *carotovora* strains were distributed into 11 of the 21 groups described by Darrasse et al. (1994), eight of which contained only strains of this subspecies. RFLP group 9 contained numerous *E. carotovora* subsp. *carotovora* strains, including two strains not growing at 37 °C (EII 338, 22ϵ), whereas group 4 was common to strains of *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera*. Groups 3 and 5 contained only two and one *E. carotovora* subsp. *odorifera* respectively. Five *E. carotovora* subsp. *carotovora* strains isolated from different continents and plants, belonged to 3 RFLP groups (22, 23, 24) not previously described (Figure 1). Groups 22 and 23, containing one strain each were characterised by the *Alu*I restriction sites (digestion pattern XVIII), and differed from each other by the *Hae*II and *Hpa*II restriction sites. The size of the *Alu*I restriction fragments were 253-bp, 125-bp and 49-bp. The three strains of group 24 were characterised by the absence of the *Hpa*II restriction sites (digestion pattern XIX).

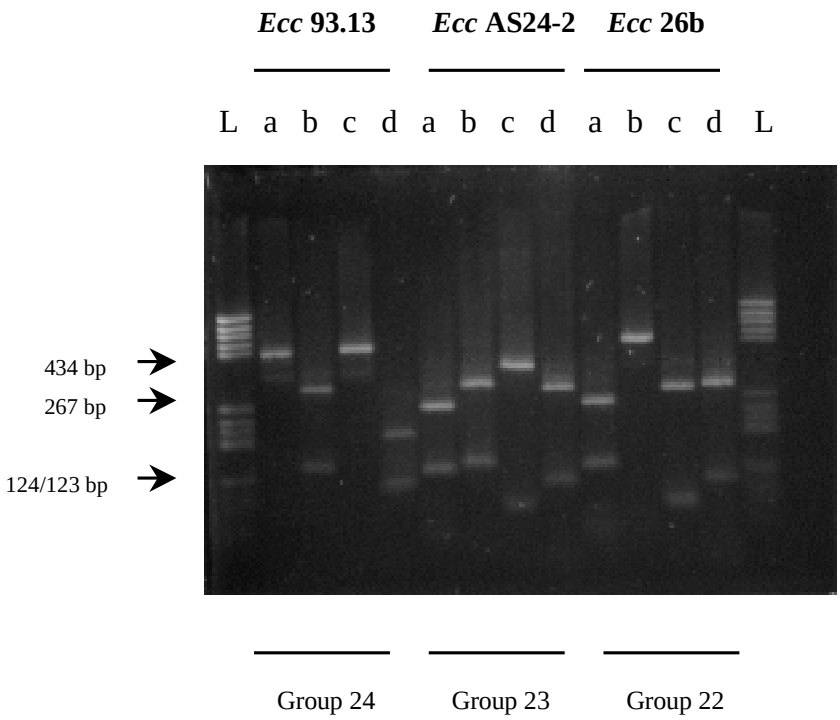

Figure 1. Agarose gels (2.7%) showing the restriction patterns by *Alu*I (a), *Hae*II (b), *Hpa*II (c), and *Sau3A*I (d) of PCR-amplified DNA from selected *Erwinia carotovora* strains corresponding to the new RFLP groups (22, 23, 24). Selected fragments of the ladder marker (L) (marker V, Boerhinger, Mannheim) are indicated in base pairs (bp) to the side of the gel.

Phenetic and cladistic analyses

Phenetic and cladistic analyses were performed on all 24 RFLP groups described so far. The UPGMA method of clustering yielded three main domains designated I, II, and III (Figure 2). Domain I, composed of RFLP groups 1, 2 (*E. carotovora* subsp. *atroseptica*) and 21 (*E. carotovora* subsp. *wasabiae*), was clearly distant from domains II and III, although RFLP group 21 clustered far from groups 1 and 2 in domain I. RFLP group 8 containing all *E. carotovora* subsp. *atroseptica* strains growing at 37 °C and one atypical *E. carotovora* subsp. *carotovora*, not growing at this temperature, was situated in domain III. Domain II contained all the *E. carotovora* subsp. *odorifera* RFLP groups. The new groups 22 and 23 belonged to the II domain, whereas group 24 was situated in the III domain. The two strains originating from Malawi (groups 15 and 17; Darrasse et al., 1994) clustered together, clearly distant from the other groups of domain III. The only atypical *E. carotovora* subsp. *carotovora* strain from Cuba, constituting group 19 (Darrasse et al., 1994), showed an unique RFLP pattern and clustered alone, outside the 3 main domains. However, consensus trees drawn from the data generated after 1000 or 5000 bootstraps of the original 0–1 matrix showed a low percentage of stability (maximum 44%) of the different nodes (data not shown).

The three domains described above (I, II, III) were globally conserved in cladistic analysis with the Mix and Restml programs, although the two algorithms yielded a different position for some RFLP groups (Figures 3A and 3B). Overall, the best concordance was between trees generated with the UPGMA and the Restml algorithms. For instance, Restml and UPGMA clustered group 10 within the III domain, whereas Mix included it in the II domain. Mix also separated groups 2 and 1 (*E. carotovora* subsp. *atroseptica*), clustering the latter with group 21 (*E. carotovora* subsp. *wasabiae*) at a large distance while group 2 formed a branch by itself. By contrast, Restml maintained the cluster of groups 1 and 2 generated in the UPGMA. Finally, group 19, which clustered alone in the UPGMA and Restml dendrograms, was closely associated to group 20 in the Mix analysis. Again, consensus trees drawn from the data generated after bootstrapping of the original 0–1 matrix showed a low

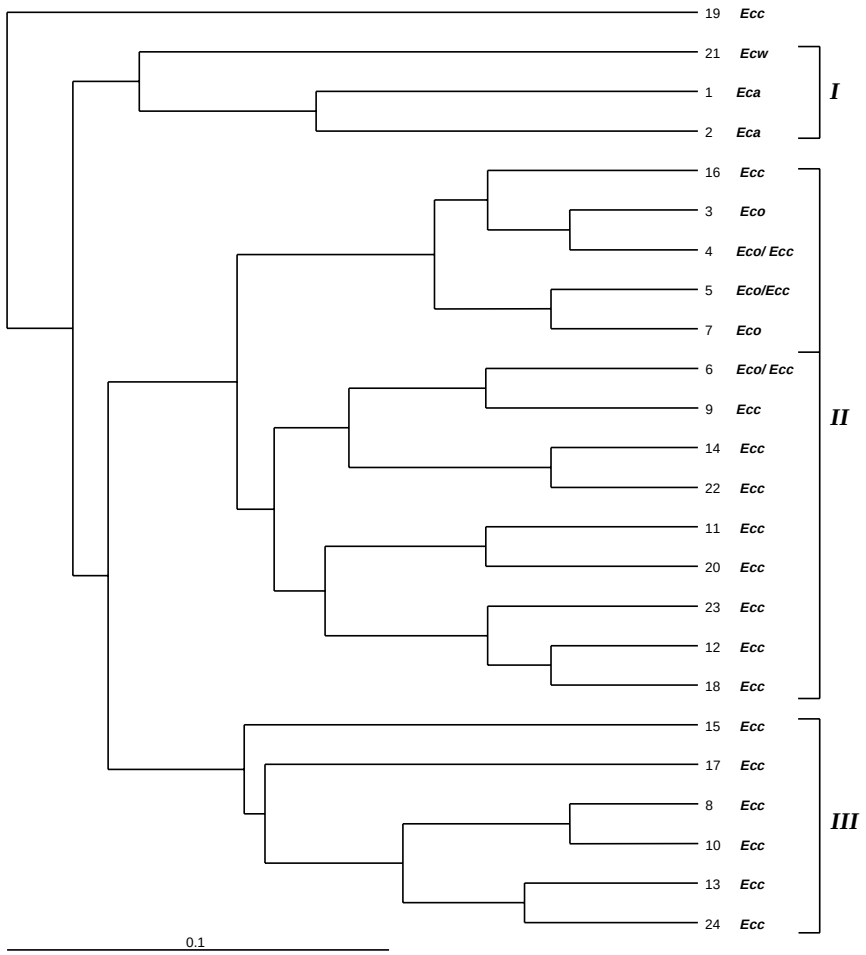

Figure 2. Phenogram obtained by UPGMA (unweighted pair-group method with average) clustering of the similarity matrix between RFLP groups detected within *Erwinia carotovora*, calculated as complements to one of Sorensen-Dice similarity coefficients.

percentage of stability of the different nodes (maximum 39% in Mix, 200 bootstraps; maximum 44% in Restml, 100 bootstraps; data not shown).

Detection thresholds of E. carotovora *subsp.* atroseptica *in various substrates*

PCR performed on samples following a 48 h enrichment step was the most sensitive technique for detecting the bacterium in plant extracts (leaves, stems and tuber peel), wash water, and soil extracts. The enrichment step improved PCR sensitivity by a factor 10^2 to 10^4 depending on the substrate considered (Table 3). DAS-ELISA and IMS-PCR were equivalent, and ca. 10^3 times less sensitive than the PCR enrichment technique. All techniques proved most sensitive when applied to plants extracts. Detection thresholds were also consistently lower in soil extracts than in wash waters. Bacteria could not be detected in wash water or in soil by IMS-PCR.

Discussion

Methodological improvements to the PCR-RFLP protocol

A simple and rapid protocol, useful for characterising *E. carotovora* strains by PCR-RFLP without extracting DNA, permitted amplification of DNA from 109 of the 140 strains included in this study. The remaining 31 strains, belonging to *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *betavasculorum* and *E. carotovora* subsp. *wasabiae* did not yield amplification products after simply boiling the bacterial suspensions before

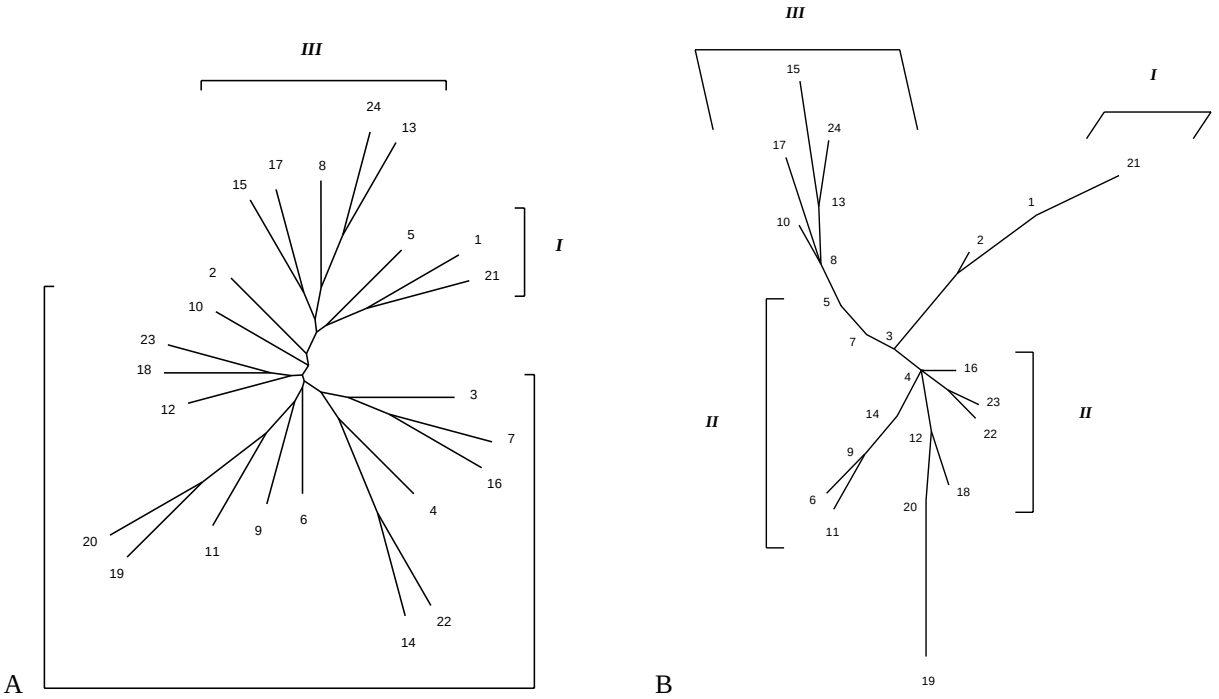

Figure 3. Unrooted cladograms showing the relationships between RFLP groups within *Erwinia carotovora*, generated with the Mix (Fig. 3A) or the Restml (Fig. 3B) modules of the PHYLIP package.

Table 3. Detection thresholds (bacteria per ml of analysed sample) of molecular and serological detection techniques applied to plant, soil and water samples artificially contaminated with *Erwinia carotovora* subsp. *atroseptica*

Detection technique	Enrichment step	Leaves	Stems	Peels	Washing-Water	Soil
PCR	0 h	$6\ 10^6$	$6\ 10^6$	$6\ 10^5$	$4\ 10^9$	$4\ 10^9$
	48 h	$1.5\ 10^3$	$1.5\ 10^2$	$1.5\ 10^3$	4.10^7	4.10^5
DAS-ELISA	0 h	$8\ 10^5$	$8\ 10^5$	$8\ 10^5$	$2\ 10^7$	$2\ 10^7$
	48 h	$8\ 10^5$	$8\ 10^5$	$8\ 10^5$	$2\ 10^7$	$2\ 10^6$
IMS-PCR	0 h	$1.5\ 10^6$	$1.5\ 10^8$	$1.5\ 10^6$	$> 4\ 10^9$	$> 4\ 10^9$

the PCR, but, with the exception of *Erwinia carotovora* subsp. *betavasculorum*, gave good amplification after either addition of Tween 20 to the suspension prior to boiling or DNA extraction. This indicated that the use of a simple protocol, although functional in most instances, can sometimes lead to false negative results if applied routinely for detection or characterisation purposes. Therefore, for these applications, any sample giving no amplification after boiling should be tested again with more efficient DNA extraction protocols. Extraction procedures using a Tris-EDTA buffer containing ca 1% (wt/vol) sodium dodecyl sulfate gave good results when applied on plant samples (De Boer and Ward, 1995; Fraaije et al., 1996; Fréchon, pers. com.). The same buffer could be used for bacterial lysis of pure cultures. Sodium hydroxide 0.05M could also replace ultrapure water containing Tween 20 to improve cell lysis efficiency during the ten min of boiling the bacterial suspension (Elphinstone et al., 1998).

Diversity and relationships within and among E. carotovora *subspecies*

Extending the collection to numerous strains originating from countries, hosts or isolation periods not

previously investigated revealed a greater diversity than that already observed within *E. carotovora* subsp. *carotovora* (Darrasse et al., 1994), as evidenced by the determination of three new RFLP groups within this subspecies. Twenty-one of the 24 groups identified so far among *E. carotovora* proved to belong to one of these subspecies, as indicated by biochemical characterisation (groups 1 and 2 for *E. carotovora* subsp. *atroseptica*, group 21 for *E. carotovora* subsp. *wasabiae*, groups 3 and 7 for *E. carotovora* subsp. *odorifera*, and groups 8-20 and 22-24 for *E. carotovora* subsp. *carotovora*). Only three groups (4, 5, 6) contained strains belonging to either *E. carotovora* subsp. *carotovora* or *E. carotovora* subsp. *odorifera*.

PCR-RFLP data also clarified the status of strains with atypical biochemical features, such as the capacity of some strains of *E. carotovora* subsp. *atroseptica* to grow at 37 °C (Thomson et al., 1981). Priou (1992) found that *E. carotovora* subsp. *atroseptica* strains able to grow at 37 °C were phenetically related to *E. carotovora* subsp. *odorifera*, based on a combination of their biochemical features and isoenzyme profiles. However, phenetic analyses of RFLPs of rRNA and of pectinases-encoding genes showed these strains to be related to *E. carotovora* subsp. *carotovora*. Futhermore, the fact that their fatty acid profiles were intermediate between those of *E. carotovora* subsp. *atroseptica* and of *E. carotovora* subsp. *carotovora* and that they cross-reacted with *E. carotovora* subsp. *carotovora* antibodies (Priou, 1992) seems to demonstrate that those strains are closer to the *carotovora* than to the *atroseptica* or *odorifera* subspecies. This hypothesis was further strengthened by the PCR-RFLP data which included them an *E. carotovora* subsp. *carotovora* group (group 8) (Darrasse et al., 1994). The four *E. carotovora* subsp. *atroseptica* strains growing at 37 °C (78a, 4b, 65a, 2a) in our collection also fell into RFLP group 8, suggesting that these strains were similar to those tested by Priou (1992) and Darrasse et al. (1994). The absence of PCR amplification (data not shown) using primers Y45 and Y46, developed by Fréchon et al. (1995) and specific for *Erwinia carotovora* subsp. *atroseptica*, confirmed that these strains do not belong to the *atroseptica* subspecies. Conversely, the three *E. carotovora* subsp. *carotovora* strains unable to grow at 37 °C (EII 338, EII 354, 22ϵ), similar to those described by De Boer and McNaughton (1987) and by Priou (1992), fell into the RFLP groups 8 and 9, specific to *E. carotovora* subsp. *carotovora*. Moreover, five *E. carotovora* subsp. *carotovora* isolates (67c, EII135, EII338, EII352, and EII 354), identified as such on the basis of their biochemical properties and of their association with RFLP groups 8 or 9, were shown to produce indole. Although indole production is commonly accepted as one of the key properties differentiating *E. carotovora* from *E. chrysanthemi* (Verdonck et al., 1987), our results, as well as those of De Boer and McNaughton (1987) and Karnjanarat et al. (1987) on other strains, invalidate its discriminating value for the two species. Overall, our data showed that biochemical tests generally allow an accurate identification of *E. carotovora* subspecies, but should be associated with other identification tools for strains showing apparent inconsistencies between biochemical traits.

The different RFLP groups, including the three new ones, clustered in an UPGMA phenogram similar to the one described by Darrasse et al. (1994), whose B and C domains corresponded globally to our domains II and III with the addition of the new RFLP groups 22 and 23 to domain B and of group 24 to domain C. RFLP groups belonging to domain III, which are more precisely characterised by the *Sau*3*A*I profile IV, included only *E. carotovora* subsp. *carotovora* strains originating from various hosts in tropical or mediterranean climates. However, other *E. carotovora* subsp. *carotovora* strains isolated from warm climates clustered in domain II, thus not supporting a relationship between the PCR-RFLP classification of the *carotovora* subspecies and strict geographical distribution. Nevertheless, RFLP group 19, constituted of the only two strains originating from Cuba (Darrasse et al., 1994), appeared as a distant and separated cluster. It would be interesting to investigate further the possible existence of a particular population of the subspecies in Cuba.

Molecular groupings were also loosely related to biological or geographical origins in the other *E. carotovora* subspecies, even those with restricted host and geographical ranges. For instance, *E. carotovora* subsp. *odorifera* appeared as a homogeneous subspecies considering its limited host range and geographical distribution, but the 18 strains tested (including those tested by Darrasse et al., 1994) were distributed into five RFLP groups. However, RFLP groups 3 and 7, specific to *E. carotovora* subsp. *odorifera* and clearly identified by the association of the *Hae*II profile VI and *Hpa*II profile XVI, were characterised by some host specialisation, all eight strains being isolated from Umbelliferae or monocotyledons. Similarly, the ten *E. carotovora* subsp. *odorifera* strains

from RFLP groups shared with strains of *E. carotovora* subsp. *carotovora* (groups 4, 5 and 6) were isolated from Compositae, with the exception of strain CFBP 3261 from leek. It should however be noted that the *E. carotovora* subsp. *carotovora* strains isolated from Compositae were not restricted to RFLP groups 4, 5 and 6. This indicates that there is only a weak association between host ranges and molecular groupings. Finally, the coexistence of *E. carotovora* subsp. *atroseptica* isolates from potato and tomato in RFLP group 2 could be explained by a progressive adaptation of tomato isolates to potato. This hypothesis is supported by the fact that those strains were shown to be pathogenic to potato (Priou, 1992). A larger number of isolates from tomato should now be tested to confirm their exclusive association with this RFLP group.

The phenetic and cladistic analyses unveiled some relationships between *E. carotovora* subspecies, although the markers used proved ill-suited to a proper phylogenetic analysis. The low stability of the consensus trees generated by resampling the data was probably due to the large extent of PCR-RFLP polymorphisms relative to the number of markers used, and to the common occurrence of rare alleles. However, the phenograms produced by UPGMA and Restml were in good agreement, and supported the hypothesis of a recent divergence between *Erwinia carotovora* subspecies. The phenograms stressed the phylogenetic proximity of the two most diverse subspecies, *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp *odorifera* subspecies, confirming previous results (Gallois et al., 1992; Priou, 1992). Our findings also confirmed the distinctness of *E. carotovora* subsp. *atroseptica*, which appeared as an homogeneous subspecies with high host specialisation, suggesting a clonal structure within this subspecies. This hypothesis is supported by previous work on RFLPs of pectinase encoding genes and rRNA genes of *E. carotovora* (Priou, 1992), and by analyses of DNA homology (Gallois et al., 1992). *E. carotovora* subsp. *atroseptica* clustered with *E. carotovora* subsp. *wasabiae* strains in the same phenon, in accordance with previous cladistics analyses (Darrasse et al., 1994; Bouchek, 1994); however, even though these two subspecies were closer to each other than to any other group, they were distant enough to appear distinct.

Recent work based on DNA relatedness within *E. carotovora* supported elevation of *atroseptica*, *betavasculorum* and *wasabiae* subspecies to species status (Samson et al., 1998). The distinctness of *E. carotovora* subsp. *betavasculorum* from the other subspecies, evidenced by the absence of an amplified fragment after PCR, supports the proposal by Samson et al. (1998) of a specific denomination for this group of strains.

Detection methodology and potential applications

When coupled to a 48 h enrichment step, PCR proved sensitive for detecting *E. carotovora* subsp. *atroseptica* in artificially infected plant extracts, and allowed detection of this bacterium in soil and water extracts. Detection was achieved using primers Y1–Y2, specific for *E. carotovora*, on samples artificially contaminated with an *E. carotovora* subsp. *atroseptica* strain. However, the detection thresholds determined by the technique are baseline values, since previous tests using pure cultures or extracted DNA showed that these primers performed best for detecting *E. carotovora* subsp. *carotovora* and worst for detecting *E. carotovora* subsp. *atroseptica* (Hélias, 1994). Detection thresholds for *E. carotovora* subsp. *atroseptica* could be improved from 4.10^7 to 4.10^5 bacteria/ml when primers Y1–Y2 are replaced by primers Y45–Y46 specific for this subspecies (Le Roux, 1995).

The enrichment step, which targets only living bacteria and considerably improves the detection thresholds of the various techniques, presents obvious advantages. Coupled with PCR, enrichment permitted detection of population levels (10^3 bacteria/tuber) below which blackleg is likely to develop from seed contamination (Pérombelon, 1992). The detection thresholds provided by the PCR technique in soil and water preparations remain 10 to 100 times higher than in plant extracts. These differences probably reflect the higher concentration of PCR inhibiting substances in soil and wash water samples. It could be possible to improve detection in these media by further diluting the samples. Irrespective of the substrate tested, PCR proved generally more sensitive than serological methods (DAS-ELISA or IMS-PCR), when samples submitted to the same enrichment steps were compared. The poor performance of serology can be due, at least in part, to the use of monoclonal antibodies, which have a lower affinity for the target bacteria because fixation is limited to a single epitope motif (Van der Wolf et al., 1996). However, Gorris et al. (1994) obtained detection thresholds as low as $2.4\ 10^2$ bacteria.ml^{-1} with this antibody, when processing peel extracts artificially infected with *E. carotovora* subsp. *atroseptica* with an enrichment step. Inactivation of the antibody was not responsible for the much lower performance

of 4G4 in our conditions, since detection thresholds on pure bacterial suspensions without enrichment (2 10^5 bacteria/ml) were comparable to those reported by Gorris et al. (1994). It is therefore possible that the low performance of serological detection arose from the relatively high sample dilutions (1 g of plant material in 25 ml diluent, as compared to 1 g per 2 ml in the protocol of Gorris et al., 1994). An added drawback of detection based on the monoclonal antibody 4G4, relative to PCR, is its limitation to the strains of *E. carotovora* subsp. *atroseptica* belonging to serogroup I. However, despite its limited sensitivity, DAS-ELISA could usefully complement PCR for investigating the major stages of the disease cycle, including the detection of primary inoculum sources and the modes of contamination of daughter tubers, because it allows the processing of a large number of samples without cumbersome extraction protocols.

No precise data are available to determine the levels of soil populations of *E. carotovora* in relation to disease development. Many authors consider that *E. carotovora* is short-lived in soil (e.g., Pérombelon and Salmon, 1995), but this conclusion is possibly biased by the poor performance of detection methods in soil (Meneley and Stanghellini, 1976). It is therefore possible that the detection thresholds in soil provided by the PCR technique are actually low enough to detect levels of epidemiological significance. Further work is now needed to improve the sensitivity of the technique, and to validate it in a wide range of agricultural soils. Furthermore, the tests were conducted on very small quantities of soil, raising the question of sampling procedures.

Since the primers used for the PCR reaction can amplify the DNA of the two *Erwinia* subspecies important in potato crops (*E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora*), the technique developed here can be used to provide a comprehensive study of the epidemiology of potato soft rot. However, experiments using field samples, where both subspecies can occur simultaneously, raise the difficulty of discriminating the pathogens involved. RFLP might not be suitable for this purpose, because of the large variability within *E. carotovora* subsp. *carotovora* and the lack of single bands diagnostic for this taxon. The use of primers Y1-Y2 on replicate samples, which amplify DNA from *E. carotovora* subsp. *carotovora* better than DNA from *E. carotovora* subsp. *atroseptica,* and Y45–Y46, specific for the latter subspecies and requiring the same amplification conditions as Y1–Y2, may prove helpful in solving this difficulty.

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