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Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analysis of a *recA* gene fragment

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Genotypic characterization, based on the analysis of restriction fragment length polymorphism of the *recA* gene fragment PCR product (*recA* PCR-RFLP), was performed on members of the former *Erwinia* genus. PCR primers deduced from published *recA* gene sequences of *Erwinia carotovora* allowed the amplification of an approximately 730 bp DNA fragment from each of the 19 *Erwinia* species tested. Amplified *recA* fragments were compared using RFLP analysis with four endonucleases (*Alu*I, *Hin*fI, *Tas*I and *Tru*1I), allowing the detection of characteristic patterns of RFLP products for most of the *Erwinia* species. Between one and three specific RFLP groups were identified among most of the species tested (*Erwinia amylovora*, *Erwinia ananas*, *Erwinia cacticida*, *Erwinia cypripedii*, *Erwinia herbicola*, *Erwinia mallotivora*, *Erwinia milletiae*, *Erwinia nigrifluens*, *Erwinia persicina*, *Erwinia psidii*, *Erwinia quercina*, *Erwinia rhapontici*, *Erwinia rubrifaciens*, *Erwinia salicis*, *Erwinia stewartii*, *Erwinia tracheiphila*, *Erwinia uredovora*, *Erwinia carotovora* subsp. *atroseptica*, *Erwinia carotovora* subsp. *betavasculorum*, *Erwinia carotovora* subsp. *odorifera* and *Erwinia carotovora* subsp. *wasabiae*). However, in two cases, *Erwinia chrysanthemi* and *Erwinia carotovora* subsp. *carotovora*, 15 and 18 specific RFLP groups were detected, respectively. The variability of genetic patterns within these bacteria could be explained in terms of their geographic origin and/or wide host-range. The results indicated that PCR-RFLP analysis of the *recA* gene fragment is a useful tool for identification of species and subspecies belonging to the former *Erwinia* genus, as well as for differentiation of strains within *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*.

Keywords: fingerprinting, differentiation, plant-pathogenic bacteria, recombinase A

INTRODUCTION

Several Gram-negative, non-spore-forming, facultatively anaerobic, rod-shaped bacteria have been classified traditionally into the genus *Erwinia*, mainly on the basis of their association with plants as either pathogens, epiphytes or saprophytes (Lelliott & Dickey, 1984). The species status is usually defined by biochemical and physiological tests (Verdonck *et al*., 1987). Plant pathologists have divided the strains into three groups based on numerical taxonomic analysis (Dye, 1968, 1969a, b, c). The 'carotovora' group is characterized by the ability to produce pectinolytic enzymes causing soft rot. The 'amylovora' group consists of pathogens that cause dry necrotic or wilting symptoms on their host plants. The 'herbicola' group consists of epiphytes, weak plant pathogens and saprophytes occurring in soil, water or the atmosphere, as well as in animal or human tissues. In the latter case they are classified in the genus *Enterobacter* (Beji *et al*., 1988; Lind & Ursing 1986).

Several immunological methods have been developed for the detection and identification of *Erwinia carotovora* subsp. *atroseptica* (De Boer & McNaughton; 1987; Gorris *et al*., 1994; Hyman *et al*., 1995), *Erwinia chrysanthemi* (Samson *et al*., 1989) and *Erwinia amylovora* (Vantomme *et al*., 1982). Furthermore, tests based on PCR have been described only for the more econ-

omically important *Erwinia* species: *E. carotovora* subsp. *atroseptica* (Darrasse *et al*., 1994; De Boer & Ward, 1995; Fréchon *et al*., 1998; Smid *et al*., 1995), *E. amylovora* (Bereswill *et al*., 1992, 1995; Guilford *et al*., 1996; McManus & Jones, 1995), *E. chrysanthemi* (Nassar *et al*., 1996) and Ligase Chain Reaction (LCR) for *Erwinia stewartii* (Willson *et al*., 1994). Toth *et al*. (1999a) described a one-step 16S rDNA PCR-based method for the detection of all soft rot *Erwinia* species. However, this method did not enable the identification of the species and subspecies within the genus *Erwinia*.

Recent intensive studies of 16S rDNA sequences suggest that this approach could be used for identification purposes and also to resolve the taxonomic relationships of different species and groups of *Erwinia* (Kwon *et al*., 1997; Hauben *et al*., 1998; Mergaert *et al*., 1999; Kim *et al*., 1999). As a result, it has been proposed that the genus *Erwinia* should be divided into four new genera, namely *Erwinia*, *Pectobacterium*, *Pantoea* and *Brenneria*. The genus *Erwinia* has been restricted to six species according to Hauben *et al*. (1999), *E. amylovora*, *Erwinia mallotivora*, *Erwinia persicina*, *Erwinia psidii*, *Erwinia rhapontici* and *Erwinia tracheiphila*. The resurrected genus *Pectobacterium* consists of four subspecies of *E. carotovora* together with *E. chrysanthemi*, *Erwinia cacticida* and *Erwinia cypripedii*. Six species, *Erwinia alni*, *Erwinia nigrifluens*, *Erwinia paradisiaca*, *Erwinia quercina*, *Erwinia rubrifaciens* and *Erwinia salicis*, have been classified in a new genus, *Brenneria*. Five species previously classified in the genus *Erwinia*, namely *Erwinia ananas*, *Erwinia herbicola*, *Erwinia milletiae*, *Erwinia stewartii* and *Erwinia uredovora*, have been reclassified in the genus *Pantoea*. This new nomenclature has not generally been accepted by plant pathologists; nevertheless both nomenclatures are currently in use.

Regardless of the taxonomy, it is important to identify bacterial species accurately and rapidly. New approaches based on application of several molecular markers give more information about genome specificity. As well as 16S and 23S rRNA there are several other candidates: heat-shock proteins (Hsp70, GroEL, Hsp60), the ATPase β subunit, RNA polymerases and recombinase A (RecA) can serve as molecular markers for the identification of bacterial pathogens (Ludwig & Schleifer, 1999). RecA is a multifunctional protein involved in homologous recombination, DNA repair and the SOS response (Eisen, 1995). It is considered to be universally present in prokaryotic and eukaryotic cells as it shows a high degree of sequence conservation. RecA protein and *recA* gene sequence comparisons have been used to speculate on phylogenetic relationships among genera and species (Lloyd & Sharp, 1993; Eisen, 1995; Karlin *et al*., 1995). The *recA* gene has been used for typing of acinetobacters (Nowak & Kur, 1995) and for identification of *Mycobacterium* species (Blackwood *et al*., 2000) and the *Bulkholderia cepacia* complex (Mahenthiralingam *et al*., 2000). Preliminary results showing the usefulness of *recA* PCR-RFLP for genotyping of *E. carotovora* were presented by Waleron *et al*. (2001).

This paper describes a method based on the analysis of *recA* gene polymorphism for the identification of the different species and subspecies of the former *Erwinia* genus. In addition, an analysis of the differentiation between the subspecies of *E. carotovora* and variation within *E. chrysanthemi* has been done.

METHODS

Bacterial strains. One hundred and seventy-seven strains of 19 different species belonging to the former *Erwinia* genus were tested (Table 1). In addition strains of some plant-pathogenic bacteria [*Agrobacterium tumefaciens* (3), *Pseudomonas savastanoi* pv. *phaseolicola* (4), *Xanthomonas vesicatoria* (3), *Xanthomonas hortorum* pv. *pelargonii* (1)] and of several other bacterial species [*Bacillus* (now *Geobacillus*) *stearothermophilus* (1), *Citrobacter freundii* (2), *Enterobacter* (now *Pantoea*) *agglomerans* (1), *Enterobacter sakazakii* (1), *Enterobacter cloacae* (1), *Escherichia coli* (4), *Klebsiella oxytoca* (1), *Klebsiella pneumoniae* (1), *Morganella morganii* (1), *Proteus vulgaris* (1), *Salmonella* Anantum (1), *Salmonella* Heidelberg (1), *Serratia marcescens* (1), '*Shigella shigae*' (1), *Staphylococcus aureus* (1), *Staphylococcus epidermidis* (1)] were examined.

For DNA preparation, *Erwinia*, *Agrobacterium*, *Pseudomonas* and *Xanthomonas* strains were grown overnight in tryptic soy broth (TSB; BioMérieux) in shaken culture at 28 °C. *E. chrysanthemi* and other *Enterobacteriaceae* were grown at 37 °C and *E. cacticida* at 43 °C. Cells were harvested by centrifugation and resuspended in TE buffer (50 mM Tris/HCl, 40 mM EDTA, pH 8·0). The bacterial DNA was extracted using the SDS/proteinase K based method described by Ausubel *et al*. (1992).

Primer design. Oligonucleotide primers were designed on the basis of the sequence of the *E. carotovora recA* gene described by Zhao & McEntee (1990). Regions with low variability were chosen. The sequence of each primer (5′-GGTAAAGGGTCTATCATGCG-3′ and 5′-CCTTCACCATACATAATTTGGA-3′) was checked for homology to other sequences that may also be amplified by them, in the GenBank and EMBL databases using the BLAST N program.

DNA amplification. DNA amplification was performed in 50 µl reaction volumes containing 5 µl 10× reaction buffer (Fermentas), 2·5 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP and dTTP, 20 pmol each primer, 0·1% (v/v) Tween 20, 50–100 ng DNA and 1 U recombinant *Taq* DNA polymerase (cloned and purified by Dr J. Osipiuk, Department of Microbiology, University of Gdansk). Amplification was performed using a UNOII Biometra thermocycler with initial denaturation (95 °C, 3 min), followed by 32 cycles of denaturation (94 °C, 1 min), annealing (47 °C, 1 min) and extension (72 °C, 2 min), with a final extension (72 °C, 5 min). The amplified products were electrophoretically separated in 6% (w/v) polyacrylamide gel at 120 V for 10 h in TBE buffer and visualized with UV light after staining in ethidium bromide (0·5 µg ml^{-1}).

Restriction fragment length analysis. The amplified DNA fragments were digested with four restriction endonucleases (*Alu*I, *Hin*fI, *Tas*I and *Tru*1I), which were selected on the basis of the nucleotide sequence of the *recA* gene of *E. carotovora* using Vector NTI software. The restriction analysis was performed overnight with 2·5 U of each endonuclease using the buffer and temperature recommended by the manufac-

Table 1. Origin and *recA* PCR-RFLP groups of the bacterial strains used in this study

Bacterial strain*	Host	Geographic origin and year of isolation	RFLP group
***Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*)**			
16[a]	Potato	Arizona, USA	1
22[a]	Potato	Arizona, USA	1
86[a]	Potato stem	Peru	1
1015[a]	Soil	Scotland	1
147[k]		Sweden	1
SAC 12[e]		Finland	1
185[d]		Germany, 1986	1
16 A-1[t]	Potato stem	Poland, 1996	1
17 A-1[t]	Potato stem	Poland, 1996	1
7 A-1[t]	Potato stem	Poland, 1996	1
1043[a]	Potato stem	Scotland	2
324[b]	Potato	Greece, 1971	2
75 B-1[t]	Potato tuber	Poland, 1996	2
***Erwinia carotovora* subsp. *carotovora* (*Pectobacterium carotovorum* subsp. *carotovorum*)**			
ATCC 15713[Ti]	Potato	Denmark	3
PH 200[e]	Potato stem	Finland	3
103[a]	Potato tuber	Scotland	3
140[a]	Potato	Arizona, USA	3
142[a]	Potato	Arizona, USA	3
1 A-1[t]	Potato stem	Poland, 1996	3
15 A-3[t]	Potato stem	Poland, 1996	3
16 A-2[t]	Potato stem	Poland, 1996	3
NAP 120/408[j]	Sunflower	Hungary, 1986	4
116[a]	Swede	Scotland, 1977	4
142[a]	Potato	Arizona, USA	4
156[a]	Potato stem	Tasmania, 1973	4
536/96[f]	Cabbage	Poland, 1996	4
539/96[f]	Leek	Poland, 1996	4
545/96[f]	Celery	Poland, 1996	4
548/96[f]	Carrot	Poland, 1996	4
AH2[g]		Oregon, USA	4
135[n]	Potato	Germany	4
56 A-15[t]	Potato stem	Poland, 1996	4
95[n]	Cabbage	Germany	5
137[a]	Potato	Arizona, USA	5
143[a]	Potato	Tasmania	5
793[b]	Hyacinth tuber	Greece, 1981	5
LA 128[c] (7)		USA	5
JA 024/319[j]	Hyacinth tuber	Hungary, 1986	5
8982[l]		Ukraine	5
23 A-4[t]	Potato stem	Poland, 1996	5
66 A-1[t]	Potato stem	Poland, 1996	5
160[a]	Potato stem	Tasmania, 1970	6
161[a]	Potato stem	Tasmania, 1970	6
248[a]	Soil water	Scotland, 1982	6
171[a]	Potato	Tasmania, 1970	7
180[a]	Potato tuber	Peru, 1979	7
174[a]	Potato tuber	Peru, 1977	8
791[b]	Cabbage	Greece, 1981	8
332[k]		Sweden	8
176[a]	Potato stem	Peru, 1977	9
178[a]	Potato stem	Peru, 1978	10

Table 1 (*cont.*)

Bacterial strain*	Host	Geographic origin and year of isolation	RFLP group
179[a]	Potato root	Peru, 1979	11
249[a]	Potato tuber	Scotland, 1982	12
582[d]	Chicory	Switzerland, 1985	13
MN 12-2[t]	Parsley	Poland, 1999	13
MN 6-1[t]	Carrot	Poland, 1999	13
MN 6-2[t]	Carrot	Poland, 1999	13
Re-4[t]	Celery	Poland, 1999	13
Re-5[t]	Carrot	Poland, 1999	13
MN 10-1[t]	Onion	Poland, 1999	13
134 A-2[t]	Potato stem	Poland, 1996	14
537/96[t]	Cabbage	Poland, 1999	15
159[b]	Cyclamen	Greece, 1967	16
333[b]	Artichoke	Greece, 1971	17
573[d]	Cabbage	Switzerland, 1983	17
JL 1105 (SR 319)[c]	Soil		18
43B-1[t]	Potato tuber	Poland, 1996	18
LMG 2404[e]	Potato	Denmark	19
143 A-1[t]	Potato stem	Poland, 1997	20
143 B-1[t]	Potato tuber	Poland, 1997	20
***Erwinia carotovora* subsp. *betavasculorum* (*Pectobacterium carotovorum* subsp. *betavasculorum*)**			
ATCC 43762[Ti] (= CFBP 2122[u], CU 0343[p], 479[a], LMG 2466, NCPPB 2795)	Sugar beet	California, USA, 1972	21
LA 129 (= Ecb 168)[o]	Sugar beet	USA	21
CFBP 2121[u] (= LMG 2463)	Sugar beet	USA, 1974	21
CFBP 1520[u]	Sunflower	Mexico	21
SF142,2[u]	Artichoke	La Reunion, 1986	21
CU 0307[p]	Sugar beet	Washington, USA	21
CU 0341[p] (= LMG 2462, NCPPB 2793)	Sugar beet	California, USA, 1974	21
CU 0342[p] (= NCPPB 3075)	Sugar beet	California, USA	21
CU 0350[p]	Sugar beet	Arizona, USA	21
***Erwinia carotovora* subsp. *odorifera* (*Pectobacterium carotovorum* subsp. *odoriferum*)**			
CFBP 1878[u] (= LMG 17566[T], NCPPB 3839)	Celery	France, 1979	22
482[a] (= NCPPB 3840)	Witloof chicory	France	22
487[a] (= 353)	Witloof chicory	France	22
CFBP 1880[u]	Witloof chicory	France, 1979	22
CFBP 1959[u]	Witloof chicory	France, 1980	22
CFBP 1893[u]	Celery	France, 1976	22
CFBP 3260[u] (= 1654,1)[u]	Leek	France, 1980	22
CFBP 3259[u] (= 1646,2)[u]	Leek	France, 1980	22
CFBP 1879[u]	Celery	France, 1979	22
CFBP 1892[u]	Celery	France, 1981	22
CFBP 2154[u]	Celery	France, 1982	22
CFBP 2155[u]	Celery	France, 1983	22
***Erwinia carotovora* subsp. *wasabiae* (*Pectobacterium carotovorum* subsp. *wasabiae*)**			
ATCC 43316[Ti] (= 481[a], CFBP 3304[u], NCPPB 370, SR 91)	Horseradish	Japan	23
917[a] (= NCPPB 3702)	Horseradish	Japan	23
918[a] (= NCPPB 3703)	Horseradish	Japan	23
919[a] (= NCPPB 3704)	Horseradish	Japan	23
CFBP 3308[u] (= ICMP 9125)	Horseradish	Japan, 1985	23

Table 1 (*cont.*)

Bacterial strain*	Host	Geographic origin and year of isolation	RFLP group
Erwinia chrysanthemi (***Pectobacterium chrysanthemi***)			
ET 3[y]	Tomato	Martinique, 1987	33
1240[y]	Carnation	Denmark, 1956	33
0600[a]	*Chrysanthemum*	Holland	33
0174[a]	Saintpaulia	Germany	34
1260[a]	Potato	Germany	34
4061[a]	Colocasia	Solomon Islands	34
564[b]	Carnation	Greece, 1976	35
0872[z] (= PD 484)	Potato	Holland	35
1086[z]	Potato	Finland	35
1076[z]	Potato	Spain	35
877[z]	Potato	Holland	35
1306[z]	Potato	Spain	35
9[d]		USA	36
260[d]		USA	36
4069[a] (= B 374)	*Pelargonium*	Comoro Island	37
ATCC 11663[Ti] (= NCPPB 402)	*Chrysanthemum*	USA, 1956	38
3262[y]	Chicory	France, 1981	38
1891[y]	Tobacco	USA	39
946[z]	Potato	Holland	40
996[z]	Potato	Holland	41
4078[a] (= 1271)	Corn	Egypt, 1961	42
SF109-1[y]	Sunflower	France, 1986	43
SH 230[y] (= C 413)	Tomato	Cuba	44
998[z]	Potato	Holland	45
4062[a]	*Agalonema*	St Lucia	46
0597[z]	Potato	Peru	47
Erwinia cacticida (***Pectobacterium cacticida***)			
72-1[w] (= ICMP 7452-81, ICPB EC188)	*Opuntia*	Arizona, USA, 1958	29
CFBP 3628[Td] (= ATCC 49489)	*Carnegiena gigantea*	Arizona, USA, 1958	30
DU89-8,1[w] (= ATCC 49483)	*Opuntia*	Australia, 1989	30
66-50[w] (= ICPB EC290)	*Carnegiena gigantea*	Arizona, USA, 1966	30
Texas29[w]	*Opuntia*	Texas, USA, 1971	30
Texas28[w] (= ICPB 296)	*Opuntia*	Texas, USA, 1971	30
78-28[w] (= ATCC 49482, ICPB EC283)	*Stenocereus gummosus*	Mexico, 1978	30
106[w] (= ICPB EC189)	*Carnegiena gigantea*	Arizona, USA, 1958	30
NCPPB 671[w] (= Dye EH4)	*Carnegiena gigantea*	Arizona, USA, 1980	30
DU 89-5,1[w]	*Opuntia*	Australia, 1989	31
DU 89-7,3[w]	*Opuntia*	Australia, 1989	31
66-19-1[w]	*Opuntia*	Arizona, USA, 1966	31
62-63[w] (= ATCC 49485)	*Opuntia*	Arizona, USA, 1962	31
623-2[w]	*Carnegiena gigantea*	Arizona, USA, 1959	31
88-6[w]	*Carnegiena gigantea*	Arizona, USA, 1988	31
Erwinia cypripedii (***Pectobacterium cypripedii***)			
ATCC 29267[Ti] (= CU 5464[p], NCPPB 3004, CFBP 3613[d], PDDCC 0695)	Orchid	California, USA, 1950	24
CU 5465[p]	Orchid	California, USA, 1950	24
440[a] (= NCPPB 752)	Orchid	California, USA, 1950	24
Erwinia amylovora			
ATCC 15580[Ti] (= LMG 2024[e])	Pear	UK	32
394[x]	Pear	Poland	32
661[x]	Rowan	Poland	32
691[x]	Apple	Poland	32

Table 1 (*cont.*)

Bacterial strain*	Host	Geographic origin and year of isolation	RFLP group
1056[b] (= BPIC 1989)	Quince	Greece, 1987	32
1628[b] (= BI 1990)	Quince	Bulgaria, 1990	32
G-7[s]	Pear or apple	Canada	32
E 7001M[s]	Mountain ash	Canada	32
G-5[s]	Pear or apple	Canada	32
Erwinia persicina			
ATCC 35998[Ti] (= LMG 11254, CFBP 3622[d])	Tomato	Japan	49
ATCC 756[o]	Human	Texas, USA	49
Erwinia rhapontici			
421[a] (= NCPPB 139)	Rhubarb	UK, 1940	21
422[a] (= ATCC 29284, LMG 3292, NCPPB 1739)	Rhubarb	UK, 1965	21
ATCC 29283[Ti] (= CFBP 3618[d], NCPPB 1578[e], 4240[p], LMG 2688)	Rhubarb	UK, 1963	53
Erwinia mallotivora			
CFBP 2503u (= ATCC 29573T, LMG 2708)	*Mallotus japonicus*	Japan, 1975	55
Erwinia psidii			
CFBP 3627[u] (= ATCC 49406[T], NCPPB 3555)	Common guava	Brazil, 1982	54
Erwinia tracheiphila			
CBP 2355[u] (= ATCC 33245[T])	Cucumber	USA, 1972	57
Erwinia ananas (***Pantoea ananatis***)			
ATCC 33244[Ti] (= 485[a], LMG 2665, NCPPB 1846)	Pineapple	Brazil, 1965	25
TM2[r]	Mulberry leaves	Japan, 1993	25
Mei7[r]	Mulberry pyralid	Japan, 1993	25
INE14[r]	Rice	Japan, 1991	26
Erwinia herbicola (***Pantoea agglomerans***)			
Mei3[r]	Mulberry pyralid	Japan, 1991	27
DW1[r]	Mulberry leaves	Japan, 1991	27
ATCC 33243[Ti] (= LMG 2565)	Cereals	Canada	28
BME1[r]	Silkworm	Japan, 1994	28
MBE1[r]	Mulberry leaves	Japan, 1994	28
H1[g]			28
Erwinia milletiae (***Pantoea agglomerans***)			
ATCC 33261[Ti] (= NCPPB 2519)	*Wisteria floribunda*	Japan	28
Pantoea sp.			
MW 1[g]	*Pelargonium*	Poland, 1999	28
Erwinia stewartii (***Pantoea stewartii***)			
ATCC 8199[Ti]	Corn	USA	48
ST1[g]		Ohio, USA, 1967	48
Erwinia uredovora (***Pantoea ananatis***)			
ATCC 19321[Ti] (= LMG 2676)	Wheat	USA, 1954	25
Erwinia nigrifluens (***Brenneria nigrifluens***)			
ATCC 13028[Ti] (= 1709[b], CFBP 3616[d], LMG 2694, NCPPB564)	Walnut	USA, 1955	51
Erwinia quercina (***Brenneria quercina***)			
CFBP 367[i] (= ATCC 29281[T], NCPPB 1852)	Oak	USA, 1964	56

Table 1 (*cont.*)

Bacterial strain*	Host	Geographic origin and year of isolation	RFLP group
Erwinia rubrifaciens (***Brenneria rubrifaciens***) ATCC 29291^{Ti} (= 710^{b}, CFBP 3619^{d}, LMG 2709, NCPPB 2020)	Walnut	USA, 1963	50
Erwinia salicis (***Brenneria salicis***) ATCC 15712^{Ti} (= CFBP 802^{d}, LMG 2698)	Willow	UK, 1957	52

* Names of organisms in parentheses are those proposed by Hauben *et al*. (1998). a, I. Toth, Scottish Crop Research Institute, Invergowie, Dundee, UK; b, P. G. Psallidas, Benaki Phytopathological Institute, Kifissia, Athens, Greece; c, V. Stockwell, Oregon State University, Corvallis, OR, USA; d, K. Geider, Max-Planck-Institut für Zellbiologie, Rosenhof, Germany; e, H. Saarilahti, University of Helsinki, Helsinki, Finland; f, M. Guranowska, Collection of Plant Pathogens of the Institute of Plant Protection, Poznan, Poland; g, D. Coplin, Ohio State University, Columbus, OH, USA; i, S.-W. Kwon, National Institute of Agricultural Science & Technology, RDA, Suwon, Korea; j, J. Németh, Plant Health and Soil Conservation, Station of County Baranya, Pécs, Hungary; k, P. Person, Swedish University of Agricultural Sciences, Uppsala, Sweden; l, B. Sharga, Uzhgorod State University, Ukraine; m, Ch. Wegener, Federal Centre for Breeding Research on Cultivated Plants, Groß Lüsewitz, Germany; n, M. Nachtigall, Federal Centre for Breeding Research on Cultivated Plants, Germany; o, C. M. O'Hara, Centers for Disease Control and Prevention, Atlanta, GA, USA; p, S. V. Beer, Cornell University, Ithaca, NY, USA; r, K. Watanabe, National Institute of Sericulture and Entomological Science, Tsukuba, Ibaraki, Japan; s, D. M. Hunter, Tree Fruits Agriculture & Agri-Food Canada, Southern Crop Protection & Food Research Centre, Canada; t, Department of Plant Protection and Biotechnology, Intercollegiate Faculty of Biotechnology, Gdansk, Poland; u, L. Gardan, Institute de la Recherche Agronomique, Centre de Recherches Agronomique d'Angers, Angers, France; w, T. Orum, University of Arizona, Tuscon, AZ, USA; x, P. Sobiczewski, Institute of Pomology, Skierniewice, Poland; y, Y. Bertheau, Institut National de la Recherche Agronomique, Paris, France; z, J. M. van der Wolf, Plant Research International, Wageningen, The Netherlands.

turers (Fermentas). Restriction fragments were separated in a 12 % (w/v) polyacrylamide gel at 120 V for 10 h in TBE buffer and visualized with UV light after staining in ethidium bromide (0·5 μg ml^{-1}).

RESULTS

DNA isolated from the cells of the bacteria of 19 former *Erwinia* species and five subspecies of *E. carotovora*, three other genera of plant-pathogenic bacteria (*Agrobacterium tumefaciens*, *Pseudomonas savastanoi* pv. *phaseolicola*, *Xanthomonas* spp.) and 16 other species of bacteria, mainly from the family *Enterobacteriaceae*, were used as a target in PCR reactions with primers designed to be complementary to the *E. carotovora recA* gene. The primers generated amplification products of approximately 730 bp with target DNA isolated for all of the *Erwinia* strains. At least one PCR product for each species and subspecies was sequenced directly in both directions and the presence of the specific restriction sites was confirmed (M. Waleron, K. Waleron & E. Łojkowska, unpublished results).

PCR products were digested by four restriction endonucleases (*Alu*I, *Hin*fI, *Tas*I and *Tru*1I) in four separate reactions (Fig. 1). The smallest differentiation of RFLP patterns was observed with *Hin*fI, which gave only seven different patterns with all *Erwinia* strains (Fig. 1a). The greatest differentiation was obtained after digestion of the PCR product with *Tas*I, which gave 31 patterns (Fig. 1b). *Alu*I and *Tru*1I gave 10 and 14 RFLP patterns, respectively (Fig. 1c, d). The results of the *recA* RFLP analysis of PCR product based on the number of bands and their position revealed the presence of 57 different *recA* combined RFLP patterns (restriction groups) (Table 2). The combined patterns of the restriction analysis of the *recA* gene were consistently different and characteristic for most of the species and subspecies tested (Table 1).

No amplification products were obtained from plant-pathogenic bacteria other than *Erwinia*. Of the other bacteria, only the DNA isolated from cells of bacteria belonging to *Enterobacteriaceae* (*Shigella* spp., *Serratia* spp. and *Klebsiella* spp.) yielded a product of similar size to that amplified from *Erwinia* species. However, restriction patterns were clearly different to those obtained for *Erwinia* species (data not shown).

Strains belonging to four species (*E. amylovora*, *E. cypripedii*, *E. persicina* and *E. stewartii*) and three subspecies of *E. carotovora* (subsp. *betavasculorum*, *odorifera* and *wasabiae*) occupied single RFLP groups based on *recA* PCR-RFLP (Table 1). In many other cases only one strain of a species was tested and examination of additional strains may reveal different RFLP patterns. Copies of the same strain obtained from different laboratories were tested and in all cases these showed the same RFLP profile (Table 1). Strains of *E. ananas*, *E. carotovora* subsp. *atroseptica*, *E. herbicola* and *E. rhapontici* occupied two different RFLP groups and those of *E. cacticida* three groups (Table 1). The highest diversity of the *recA* gene was observed among 57 strains of *E. carotovora* subsp. *carotovora* (18 groups) and 26 strains of *E. chrysanthemi* (15 groups) (Tables 1 and 2).

DISCUSSION

One hundred and seventy-seven strains of *Erwinia*, comprising 19 different species, were tested using PCR-RFLP with primers from the *recA* gene sequence. Fifty-

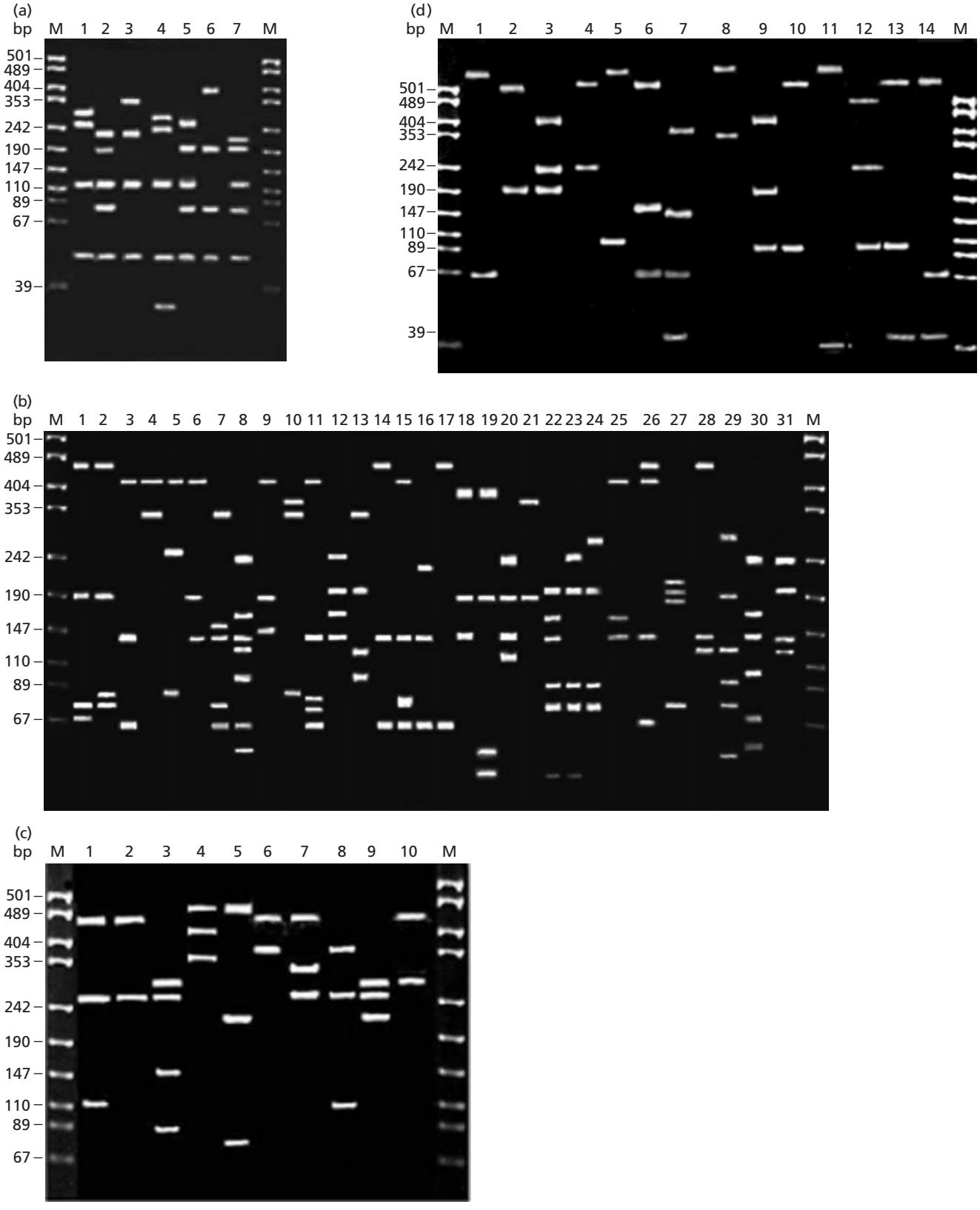

Fig. 1. Restriction analysis of a *recA* gene fragment amplified by PCR. RFLP patterns were obtained after digestion of PCR products with *Hin*fI (a), *Tas*I (b), *Alu*I (c) and *Tru*1I (d). Lane M, molecular mass size markers (pUC18/*Msp*I); other lanes, digestion products of *recA* gene fragments amplified from the genomic DNA of *Erwinia*. Lane numbers correspond to the PCR-RFLP patterns obtained for each endonuclease as shown in Table 2.

seven different RFLP groups were identified. Analysis of the results of the *recA* PCR-RFLP genotyping of 140 strains of pectinolytic *Erwinia*, reclassified by Hauben *et al*. (1998) on the basis of 16S rDNA sequence into the resurrected genus *Pectobacterium*, indicated the presence of 42 different RFLP groups (combined restriction profiles). The highest number of restriction profiles was obtained when *recA* gene fragments of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* were analysed (Table 1). A similar high level of variability in these species was observed in serological studies (De Boer & McNaughton, 1987; Samson *et al*., 1989) and in RFLP analysis of the sequences of genes encoding isoenzymes from the pectate lyase family (Darrasse *et al*., 1994; Nassar *et al*., 1996).

In contrast, the 13 strains from *E. carotovora* subsp. *atroseptica* belong to only two specific RFLP groups, 1 and 2 (Table 1). The low variability in this subspecies is in agreement with serological studies that indicated the presence of only a few serogroups (De Boer & McNaughton, 1987). Similarly, only two RFLP groups were obtained after analysis of the polymorphism of the genes encoding pectate lyase (Darrasse *et al*., 1994; Śledź *et al*., 2000). A low level of variation was also observed in PCR for enterobacterial repetitive intergenic consensus (ERIC) sequences (Toth *et al*., 1999b).

Four other pectolytic *Erwinia* species, *E. cypripedii*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *odorifera* and *E. carotovora* subsp. *wasabiae*, show only one specific combined RFLP profile each, whereas the 15 strains of *E. cacticida* give three different patterns (Table 1). This is in accordance with results reported by Alcorn *et al*. (1991) who described differences between strains of *E. cacticida* based on their physiology, pathogenicity and DNA homology.

The non-pectinolytic *Erwinia* strains, *E. amylovora* and *E. persicina*, gave one specific restriction pattern for each species, which suggests a low degree of diversity (Table 1). Although the strains of *E. amylovora* used were isolated from different *Rosaceae* plants growing in different countries, they nevertheless belonged to the same *recA* PCR-RFLP group (Table 1). The low variability among *E. amylovora* strains has also been observed in biochemical (Dye, 1968; Verdonck *et al*., 1987), serological (Vantomme *et al*., 1982), DNA hybridization (Brenner *et al*., 1974) and PFGE studies (Zhang & Geider, 1997).

For *E. rhapontici* two RFLP groups were observed. Two strains were assigned to group 21 and were identical to strains of *E. carotovora* subsp. *betavasculorum*; a single strain was in the unique group 53 (Table 1). Verdonck *et al*. (1987) reported that *E. rhapontici* strains form three groups. One of them was common for *E. rhapontici* and *E. carotovora* strains. However, in biochemical and DNA hybridization tests, the type strain of *E. rhapontici* was very similar to the strains of *E. persicina* (Hao *et al*., 1990). Phylogenetic analyses of *Erwinia* species based on 16S rDNA sequences also confirm these observations (Kwon *et al*., 1997; Hauben *et al*., 1998).

Analysis of the *recA* gene in species which have been reclassified in the genus *Pantoea* (*E. ananas*, *E. herbicola*, *E. milletiae*, *E. stewartii* and *E. uredovora*) indicated the presence of five RFLP patterns, some common to more than one species (Table 1). Only *E. stewartii* strains belonged to one RFLP group. *E. ananas* strains belonged to two different RFLP groups, 25 and 26, but group 25 also included a single strain of *E. uredovora* (Table 1). This is in agreement with the results of DNA–DNA hybridization (Mergaert *et al*., 1993) and analysis of 16S rDNA (Kwon *et al*., 1997; Kim *et al*., 1999), indicating a high degree of similarity between the two species. On that basis, these two pathogens have been described as being different pathovars of the new species *Pantoea ananas*, pv. *ananas* and pv. *uredovora*. It is interesting to note that strains of *E. ananas* and *E. uredovora* that have been reported to be ice-nucleation-active (Watanabe & Sato, 1998) were placed into RFLP group 25, whereas the strains without this activity were in RFLP group 26.

Six strains of *E. herbicola* belong to two different RFLP groups, 27 and 28, one of which, group 28, also includes single strains of *E. milletiae* and *Pantoea* sp. (Table 1). This is in agreement with data from molecular tests (Brenner *et al*., 1984; Lind & Ursing, 1986; Beji *et al*., 1988) and phenotypic data (Mergaert *et al*., 1984; Verdonck *et al*., 1987), which suggest that *E. herbicola* and *E. milletiae* are closely related; even their names are synonymous and they have both been reclassified in the genus *Pantoea* (Gavini *et al*., 1989).

Strains of *E. nigrifluens*, *E. quercina*, *E. rubrifaciens* and *E. salicis*, which have been reclassified into the genus *Brenneria*, each gave one distinct pattern (Table 1). Numerical taxonomic analysis (Verdonck *et al*., 1987) and 16S rDNA sequence analysis (Hauben *et al*., 1998) of these species showed that they form separate homogeneous groups and are distinct from each other.

The degree of genetic diversity in the different species indicated by the number of RFLP groups obtained per species could be tentatively associated to host specificity and host range of the bacteria, and to their geographical, spatial and temporal distributions. Species exhibiting host specificity and a narrow host range in a defined climate, such as *E. amylovora* and *E. carotovora* subsp. *atroseptica*, infecting rosaceous plants and potato, respectively, would tend to be genetically homogeneous. There was probably less evolutionary pressure to adapt and diversify than in the case of species with a wide host and geographical range, such as *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* (Perombelon & Kelman, 1980), both with a large number of *recA* RFLP groups.

Nassar *et al*. (1996) showed in PCR-RFLP analysis of the genes encoding pectate lyases that at least some of their groupings could be associated with hosts and geographical regions. However, no correlation could be

Table 2. RFLP groups obtained on the basis of restriction analysis of *recA* gene fragments with four restriction endonucleases

RFLP group*	PCR-RFLP patterns for specific restriction endonucleases†				*Erwinia* species assigned	No. of strains
	*Tas*I	*Tru*1I	*Alu*I	*Hin*fI		
1	1	1	1	1	*E. carotovora* subsp. *atroseptica*	10
2	2	1	1	1		3
3	3	6	6	3	*E. carotovora* subsp. *carotovora*	8
4	5	2	2	2		11
5	4	2	2	2		9
6	4	4	0	2		3
7	3	1	3	3		2
8	4	3	2	2		3
9	3	5	3	2		1
10	3	5	3	3		1
11	3	5	3	4		1
12	4	6	4	4		1
13	6	3	0	2		7
14	4	2	1	2		1
15	6	2	2	3		1
16	4	4	2	2		1
17	6	4	2	2		2
18	3	6	2	2		2
19	4	6	2	2		1
20	4	2	2	3		2
21	8	7	2	3	*E. carotovora* subsp. *betavasculorum*/ *E. rhapontici*	9/2
22	9	3	7	5	*E. carotovora* subsp. *odorifera*	12
23	11	6	6	3	*E. carotovora* subsp. *wasabiae*	5
24	13	2	2	7	*E. cypripedii*	3
25	10	10	8	6	*E. ananas*/*E. uredovora*	3/1
26	10	10	2	6	*E. ananas*	1
27	18	0	0	1	*E. herbicola*	2
28	21	6	0	3	*E. herbicola*/*E. milletiae*/*Pantoea* sp.	4/1/1
29	22	9	2	3	*E. cacticida*	1
30	23	9	2	3		8
31	24	9	2	3		6
32	12	1	2	3	*E. amylovora*	9
33	16	2	0	2	*E. chrysanthemi*	3
34	14	2	0	2		3
35	20	1	0	3		6
36	18	1	2	4		2
37	17	2	5	4		1
38	7	1	0	3		2
39	7	2	2	3		1
40	16	1	2	4		1
41	19	2	0	2		1
42	15	2	0	2		1
43	18	7	2	3		1
44	8	2	0	2		1
45	14	3	2	2		1
46	14	2	0	4		1
47	14	2	0	3		1
48	25	11	10	6	*E. stewartii*	2
49	26	14	2	1	*E. persicina*	2
50	29	8	2	2	*E. rubrifaciens*	1

Table 2 (*cont.*)

RFLP group*	PCR-RFLP patterns for specific restriction endonucleases†				*Erwinia* species assigned	No. of strains
	*Tas*I	*Tru*1I	*Alu*I	*Hin*fI		
51	28	0	0	2	*E. nigrifluens*	1
52	27	1	6	3	*E. salicis*	1
53	30	14	2	3	*E. rhapontici*	1
54	31	13	9	3	*E. psidii*	1
55	0	6	9	3	*E. mallotivora*	1
56	17	12	10	3	*E. quercina*	1
57	23	9	2	1	*E. tracheiphila*	1

* Numbers of RFLP groups based on the combined PCR-RFLP patterns. Groups were restricted to one species, except groups 21, 25 and 28.

† Numbers correspond to RFLP patterns shown in Fig. 1. Zero indicates the absence of restriction digestion by a given endonuclease.

found in this study between RFLP groups and host plants or geographical origins of the *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains tested. The apparent genetic homogeneity in *E. amylovora* and *E. carotovora* subsp. *atroseptica* could have been magnified by the fact that both bacteria were probably relatively recently distributed across the world from their centres of origin, *E. amylovora* from North America in the 20th century (Schroth *et al*., 1974) and *E. carotovora* subsp. *atroseptica* from South America in the 16th century (Salaman, 1949). Most of the strains of these species studied in this paper originated from countries other than the centre of origin.

In contrast to *E. amylovora*, *E. cacticida*, *E. chrysanthemi*, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera*, only a small number of strains of other species have been examined in this study. This is because only a few strains are listed in culture collections and those available tend to have been isolated usually from one host plant and from one geographical area. Therefore, the presence of only a few groups per species does not necessarily signify that the species are genetically homogeneous. Only when more strains from a wider range of sources have been studied can this point be verified.

In conclusion, the *recA* PCR-RFLP method can be used to rapidly identify species and subspecies of pectinolytic *Erwinia*. It allowed identification of all of the species of the former *Erwinia* genus, including members of the genus that have not been well studied and are not identified by traditional methods.

In addition, the method is a useful tool to study species diversity in relation to host specificity, host range and geographical distribution. It would also be useful to examine the distribution of bacteria connected with the worldwide exchange of plant material.

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