Journal of Applied Microbiology 2004, **96,** 302–310 doi:10.1046/j.1365-2672.2003.02156.x

Genetic characterization of *Erwinia amylovora* strains by amplified fragment length polymorphism

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2003/0489: received 9 June 2003, revised 15 September 2003 and accepted 7 October 2003

ABSTRACT

A. RICO, A. ORTIZ-BARREDO, E. RITTER AND J. MURILLO. 2003.

Aims: *Erwinia amylovora* is one of the most important pathogens of pear and apple and is subject to strict quarantine regulations worldwide, although its patterns of dispersal are largely unknown. Previous attempts to fingerprint *E. amylovora* strains by molecular techniques have detected very little polymorphism because of the high genetic homogeneity of this bacterium. Our aim was to establish and test a typing method to quantify genetic diversity among strains of this plant pathogen.

Methods and Results: Twenty-two strains from different hosts and geographical locations were examined by PCR fingerprinting with four primers and by amplified fragment length polymorphism (AFLP) with four selected combinations of primers with a single base extension. PCR fingerprinting revealed little polymorphism producing the same amplification patterns for 17 strains, while the combined AFLP patterns yielded 78 polymorphic bands (34% of total bands) and allowed the differentiation of all but two strains. Clustering of strains in the resulting dendrogram was not correlated with host, year or country of isolation, and questions previous genealogies based on PFGE patterns.

Conclusions: The AFLP technique allowed the detection of an unprecedented number of genetic markers in *E. amylovora* and proved to be the most useful tool so far for discriminating among strains of this pathogen. The results obtained in this study strongly suggest the occurrence of multiple introductions of the pathogen in Spain and other European countries.

Significance and Impact of the Study: A major limitation in understanding the ecology of fire blight is the lack of typing techniques with a high power of discrimination. This study demonstrates the high resolution and the usefulness of the AFLP technique to differentiate among *E. amylovora* strains.

Keywords: DNA fingerprinting, fire blight, genetic diversity, pathogen dissemination, PFGE, rep-PCR.

INTRODUCTION

Erwinia amylovora (Burrill) Winslow *et al.* is the casual agent of fire blight, a destructive bacterial disease that affects principally apple and pear, and other rosaceous plants of economic importance, including other fruit trees and ornamentals (van der Zwet and Keil 1979). The disease originated in North America but has spread to many parts of the world reaching New Zealand (1919), Britain (1958) and almost all of Europe (Bonn and van der Zwet 2000). Fire blight is present in a number of Mediterranean countries including Egypt, Turkey, Greece and Israel. Two important outbreaks occurred in Italy (Finelli *et al.* 1996) and in Northern Spain, in 1995, in a cider-apple orchard in Guipúzcoa, near the border with France (de la Cruz-Blanco 1996; López *et al.* 1999a). The disease was progressively detected in provinces near the first outbreak, such as

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Navarra, and in other parts of Central Spain (López *et al.* 1999b, 2002). The rapid implementation of eradication measures in Spain helped to prevent the exponential increase in the number of outbreaks that were previously observed in other countries (Bonn and van der Zwet 2000). However, fire blight remains a serious threat to the Spanish apple and pear industry. One of the main problems hampering the efficient application of appropriate preventive control methods is that modes of entry of fire blight into a new country, and its subsequent early spread, often remain a matter for speculation. Long distance dispersal of the pathogen is thought to result from transport of infected plant material, that can be asymptomatic (Thomson 2000). At shorter distances, once the inoculum is present in an orchard, the disease spreads rapidly under favourable conditions of temperature and humidity. However, many of the details of the transmission and dispersal processes remain unclear because of our current inability to discriminate among different strains of the pathogen.

E. amylovora is a genetically homogeneous species (Vanneste 1995; Momol and Aldwinckle 2000). In the past, no characteristics have been found that can distinguish strains of different geographical origins, or strains that have been isolated either from different host plants or at different times. This homogeneity has been confirmed with biochemical, serological and host range studies (Vantomme *et al.* 1982, 1986; Momol and Aldwinckle 2000). The exception are the strains isolated from *Rubus* spp., which show a distinct host range (Beer *et al.* 1996) and could be distinguished from strains of the Maloideae group by fatty acid analysis (van der Zwet and Wells 1993) and by genetic fingerprinting (Laby and Beer 1992; McManus and Jones 1995; Beer *et al.* 1996; Maes *et al.* 1996; Maes and Crepel 1997; Momol *et al.* 1997, 1999). As well, *Erwinia* strains isolated from pears in Japan can also be distinguished from the Maloideae group by pathogenic, biochemical and genetic characteristics, although their taxonomical position is not yet clear (Momol and Aldwinckle 2000; Kim *et al.* 2001). The pulse-field gel electrophoresis (PFGE) technique has revealed several restriction fragment length polymorphism (RFLPs) within the Maloideae group from various geographical origins and established several genomic patterns which grouped the strains from Europe and the Mediterranean region (Zhang and Geider 1997; Jock *et al.* 2002). These patterns are based on target variations of the endonuclease *Xba*I, which differ from one another by one or two restriction fragments. Most of the Spanish strains tested shared a PFGE pattern typical from Western France and Central Europe (Pt4), while a few strains from Central Spain (seven of 29 tested) showed a pattern from Northern France (Pt3), suggesting that at least two different introductions had occurred in Spain (Jock *et al.* 2002).

We were interested in examining a technique that could explore the complete genome and could reveal additional differences within the *E. amylovora* population. Amplified fragment length polymorphism (AFLP) analysis is a high resolution technique for fingerprinting bacteria in the study of taxonomic diversity and phylogenetic structure of bacterial populations, including plant pathogens (Janssen *et al.* 1996; Clerc *et al.* 1998; Restrepo *et al.* 1999; Savelkoul *et al.* 1999; Rademaker *et al.* 2000). The technique has proved to be comparable with DNA pairing studies and RFLPs when genetic relatedness or intrapathovar diversity is examined, and has shown more discriminatory power than random amplified polymorphic DNA (RAPD) or PCR using repetitive element primers (rep-PCR) (Clerc *et al.* 1998; Restrepo *et al.* 1999).

The objective of this study was to use rep-PCR and AFLP to determine the relatedness of 22 *E. amylovora* strains from different hosts and countries, with the aim to identify potential sources of inoculum arriving in Spain, as well as to explore the applicability of the AFLP technique to reveal the potential genotypic variability of *E. amylovora*. Our results indicate that AFLP is a highly discriminatory technique and that it could be used as a standard tool to study genotypic variation in this organism.

MATERIALS AND METHODS

Bacterial strains

The *E. amylovora* strains used in this study were kindly supplied by D. Berra (Laboratorio Agrario, Guipúzcoa, Spain), M. Borruel (Laboratorio Agrario, Navarra, Spain), M.M. López (IVIA, Valencia, Spain), and G. Sundin (Michigan State University, USA), and their characteristics are listed in Table 1. Strains were routinely propagated at 28°C using Luria–Bertani medium (LB) (Sambrook *et al.* 1989).

PCR fingerprinting

PCR fingerprinting was performed using primers ERIC1R-ERIC2 (McManus and Jones 1995), BOX (McManus and Jones 1995), IS*50* (Sundin and Murillo 1999) and the sequencing primer M13 reverse (REV; 5′-AGCGGATAACAATTTCACACAGG-3′) (Desmarais *et al.* 1998). For REV-PCR, 1X PCR buffer, 0·2 mM each dNTP, 2 mM $MgCl_2$, 10 pmol of primer and 1 U of *Taq* DNA polymerase (Bioline, London, UK) were used in the reaction. The PCR reaction consisted of a denaturation step of 10 min at 94°C followed by two cycles at 94°C for 1 min, 40°C for 5 min and 72°C for 5 min; 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and a final extension step at 72°C for 10 min. For the rest of primers

Table 1 Sources and AFLP data of *Erwinia amylovora* strains used in this study

Strain*	Host, place and year of isolation; alternative designation	AFLP data		
		Strain-specific patterns‡	Strain-specific bands‡	AFLP group
CFBP1430	*Crateaegus* sp., France, 1972	E3M1		B
Ea115	*Malus* sp., Germany,	E1M1, E2M1,		A
Ea273	*Malus domestica*, USA, 1971; obtained as CFBP3051	E1M1, E2M1, E3M1		B
EF4†	*Pyrus communis*, New Zealand, 1955; NCPPB2080	E3M1		B
ICPB EA123†	*Rubus idaeus*, USA, 1949; NCPPB2291			
IVIA1525-6	*Cotoneaster* sp., Guipúzcoa, Spain, 1996	E1M1, E3M1	E1M1	B
IVIA1614-2	*Pyracantha* sp., Segovia, Spain,1997	E2M3, E3M1		B
IVIA1731-1	*Pyrus communis*, Guipúzcoa, Spain, 1997	E3M1		B
N11†	*Pyrus communis*, USA, 1975; NCPPB2791	E1M1, E2M1, E2M3, E3M1	E2M1, E3M1	A
NCPPB311	*Pyrus communis*, Canada, 1952	E1M1		B
NCPPB1819	*Crataegus* sp., USA, 1966	E1M1, E2M1, E2M3, E3M1	E1M1, E2M3, E3M1	D
P42†	*Pyrus communis*, UK, 1958; ATCC29850, Ea595, NCPPB595			A
PB 3/142†	*Eriobotrya japonica*, Turkey, 1987; NCPPB3548	E2M3, E3M1	E2M3, E3M1	B
PV1775	*Cotoneaster salicifolius*, France	E2M3, E3M1		B
PV1778	*Cotoneaster lacteus*, France	E1M1, E2M3		A
PV1887	*Cotoneaster lacteus*, France	E1M1, E2M1, E2M3, E3M1	E2M1, E3M1	B
S59/5†	Type strain; *Pyrus communis*, UK, 1959; ATCC15580, CFBP1232, NCPPB683	E1M1, E2M1, E2M3		A
UPN500	*Pyrus communis*, Guipúzcoa, Spain, 1998	E1M1		A
UPN529	*Pyracantha* sp., Navarra, Spain, 1997			A
UPN538	*Cotoneaster* sp., Guipúzcoa, Spain, 1998	E3M1		A
UPN546	*Cydonia* sp., Navarra, Spain, 1998			A
0-288-1	USA	E1M1, E2M1, E3M1		C
8AI†	*Malus sylvestris*, The Netherlands, 1980; NCPPB3159			B

*All strains were obtained from M.M. López (IVIA), except 0-288-1, from G. Sundin (Michigan State University), UPN500 and UPN538, from D. Berra (Laboratorio Agrario, Guipúzcoa, Spain), and UPN529 and UPN546, from M. Borruel (Laboratorio Agrario, Navarra, Spain). CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; ICPB, International Collection of Phytopathogenic Bacteria, Davis, USA; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; PV, Protection des Végétaux, Angers, France; UPN, Universidad Pública de Navarra, Pamplona, Spain.

†Obtained as NCPPB strains.

‡The primer combinations that produced specific band patterns or specific bands are indicated.

the PCR reactions were performed under the conditions described for each primer (McManus and Jones 1995; Sundin and Murillo 1999). Amplifications were carried out using a Robo Cycler Gradient 96 thermal cycler (Stratagene, La Jolla, CA, USA) and separated on 1·5% agarose gels (Sambrook *et al.* 1989). Each PCR reaction was performed at least twice in separate experiments.

AFLP reactions

The AFLP procedure was performed as previously described (Vos *et al.* 1995) with slight modifications. Genomic DNA was extracted from overnight cultures in LB using a commercial kit (Puregene; Gentra Systems, Minneapolis, MN, USA) and following the manufacturer's instructions. DNA (2 μg) was digested with *Eco*RI and *Mse*I and then

Table 2 Primers used for the amplified fragment length polymorphism analysis

Adapters and Primers	Sequence
*Eco*RI-adapter	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
*Mse*I-adapter	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
*Eco*RI non selective primer	5′-GACTGCGTACCAATTC-3′
*Mse*I non selective primer	5′-GATGAGTCCTGAGTAA-3′
Selective primers	
E1	5′-GACTGCGTACCAATTC**A**-3′
E2	5′-GACTGCGTACCAATTC**T**-3′
E3	5′-GACTGCGTACCAATTC**G**-3′
M1	5′-GATGAGTCCTGAGTAA**A**-3′
M3	5′-GATGAGTCCTGAGTAA**C**-3′

ligated to the respective adapters (Table 2). The preamplification reaction was carried out with *Eco*RI and *Mse*I non-selective primers (Table 2) and consisted of 20 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s; the resulting PCR product was appropriately diluted and used as template for the selective amplification. This PCR reaction was based on a touchdown thermal profile, which consisted of 34 cycles with an initial annealing temperature at 65°C and a subsequent reduction of 1°C per cycle during nine cycles until reaching 56°C; the remaining 24 cycles were as described for the preamplification step. In all cases, *Taq* DNA polymerase from Gibco-BLR (Life Technologies, Inchinnan, UK) was used and the amplifications were carried out in a Robo Cycler Gradient 96 thermal cycler (Stratagene). After completing the selective amplification and adding 7 μl of loading buffer containing 95% (v/v) formamide, the samples were kept overnight at 4°C. The amplification products were separated on an 8% polyacrylamide denaturing gel in a Gene Reader 4200 DNA analyser (Li-COR Biosciences, Lincoln, NB, USA). Electrophoresis was carried out for 2 h 30 min at 48°C at 30 mA in 1X Tris–borate–EDTA (TBE) buffer. Amplifications were performed at least twice for each strain and only those bands that were reproducible were included in further analyses.

The number and nature of selective nucleotides can modify the complexity of the AFLP fingerprint. To select appropriate selective primers, eight *E. amylovora* strains were analysed by AFLP using the *Eco*RI and *Mse*I primers with an extension of three nucleotides (+3 primers). Primers E32 (extension AAC) and E45 (ATG) were used in combination with primers M48 (CAC), M49 (CAG), M59 (CTA), M64 (GAC), M65 (GAG) and M67 (GCA), which lead to 12 primer combinations. Twelve additional primer pairs were tested by using primers M49 and M59 in combination with primers E63 (GAA), E66 (GAT), E75 (GTA), E78 (GTT), E80 (TAC) and E81 (TAG). Finally, the entire set of strains (Table 1) was tested using six primer pairs with one selective nucleotide (E1/M1, E1/M3, E2/M1, E2/M3, E3/M1 and E3/M3) (Table 2).

Data analysis

Differential PCR or AFLP bands were scored as present (1) or absent (0) in a binary table using the Cross Checker package (version 2.9; Wageningen University, Wageningen, The Netherlands), which was used to construct a similarity matrix using the Jaccard coefficient. Cluster analysis was performed with the UPGMA method in the SAHN procedure of the NTSYS-PC program (version 2.1; Rohlf 1994) and dendrograms were constructed and plotted with the TREE option. Cophenetic value matrices were computed from the corresponding tree matrices and compared with the original dissimilarity matrix in order to estimate the goodness of fit (Rohlf 1994). A principal coordinate analysis (PCA) with the AFLP data was performed using the SIMINT, DCENTER and EIGENVECTOR procedures of the NTSYS-PC program.

RESULTS

PCR fingerprinting

For comparison of the AFLP analysis, a total of 22 *E. amylovora* strains from fruit trees and ornamental plants and one *Rubus* strain were used in a PCR fingerprinting assay using primers ERIC, BOX, IS*50* and REV. A total of 32 bands were produced by the four sets of primers, 15 of

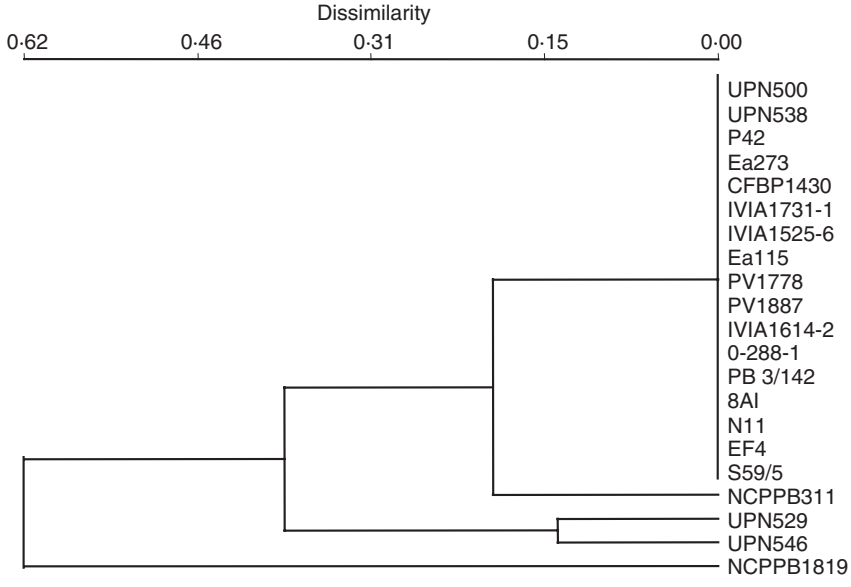

Fig. 1 Dendrogram of *Erwinia amylovora* strains based on UPGMA cluster analysis with the NTSYS-PC program using the combined PCR fingerprints with the rep-PCR primers ERIC, BOX, IS*50* and REV

which were polymorphic. The resulting dendrogram (Fig. 1), constructed only with polymorphic bands, and with a correlation coefficient of 0·99 with respect to the original dissimilarity matrix, had a limited discriminative power and most of the strains (18 of 23 tested) could not be differentiated and were grouped in the main cluster. Two Spanish strains clustered apart from the main cluster because of the presence of two specific bands obtained with primers ERIC1-2R, while strain NCPPB1819 differed from the main cluster by the presence of three bands in the REV-PCR profile. Strain ICPB EA123, isolated from *Rubus* sp., showed the greatest dissimilarity and clustered well apart from the strains isolated from pomaceous hosts, which confirms the results obtained in previous studies (Momol and Zeller 1992; McManus and Jones 1995; Maes *et al.* 1996; Maes and Crepel 1997; Momol *et al.* 1997).

AFLP analysis

A preliminary AFLP experiment with eight *E. amylovora* strains indicated that the selective amplification with +3 primers did not yield informative fingerprints in that most of the 24 combinations tested produced <10 bands, none of which were polymorphic. Among the primer pairs tested, only E45/M49, E45/M59, E63/M49, E63/M59 and E80/M59 yielded informative fingerprints with 12–30 bands per strain and several polymorphisms. Therefore, we decided to use primers with one selective base for the AFLP assays in order to increase the chances of detecting polymorphisms (Table 2). Among the six primer pairs tested, E1/M1, E2/M1, E2/M3 and E3/M1 produced a complex but readable gel pattern with 39–90 bands per strain and a total of 229 bands (Fig. 2, Table 3). None of the individual primer pairs produced a specific pattern for each strain. However, by combining the patterns of the four primer pairs all of the strains, except UPN529 and UPN546, could be differentiated. The four primers pairs produced from one to nine unique bands for five individual strains.

The 78 polymorphic bands (34% of total bands) were used to compute a similarity matrix from the Jaccard coefficient and to perform a cluster analysis (Sneath and Sokal 1973). A cophenetic matrix was computed from the resulting tree matrix and compared with the original dissimilarity matrix in order to measure the goodness-of-fit (Rohlf 1972). These matrices showed a significant correlation of 95%. Three main clusters could be clearly distinguished using 45% dissimilarity as a cut-off point (Fig. 3). Group A comprised nine strains, four of which are strains from the northeast of Spain, two from Britain, and the remaining three from France, Germany and the USA. Some of the strains grouped in this cluster showed two different PFGE patterns in previous studies (e.g. P42, Pt1 and UPN500, Pt4) (Zhang *et al.* 1998; Jock *et al.* 2002).

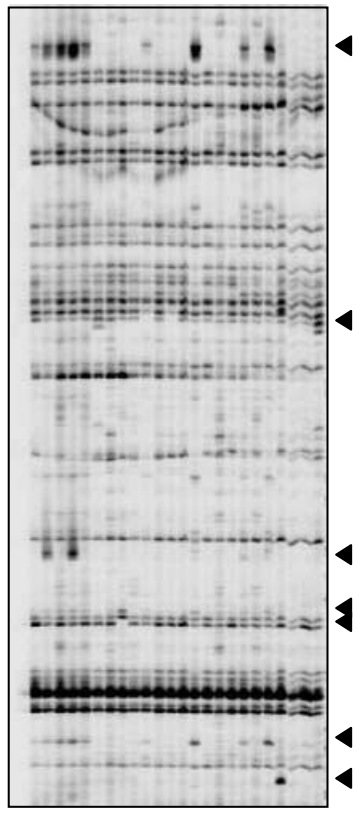

Fig. 2 Example of the AFLP patterns of *Erwinia amylovora* strains. Primer combination E1M1 was used, and only a portion of the gel is shown; the arrows on the right indicate some of the polymorphic bands observed

Table 3 Number of amplified fragment length polymorphic bands and patterns for each primer combination

Primer combination	No. of polymorphic bands	No. of patterns
E1M1	23	15
E2M1	13	11
E2M3	16	11
E3M1	26	16

Cluster B includes 12 strains from various origins, hosts and years of isolation, and some of them were previously assigned to PFGE groups (Pt3, Pt3A and Pt4) (Fig. 3) (Zhang *et al.* 1998; Jock *et al.* 2002). Strain NCPPB1819, isolated in the USA and constituting group C, is quite distant from groups A and B (>60% dissimilarity) and showed the highest number of specific bands with the three primer combinations (Table 1). Moreover, all the primer combinations produced a specific pattern for this strain, which distinguished it clearly from the rest. No obvious

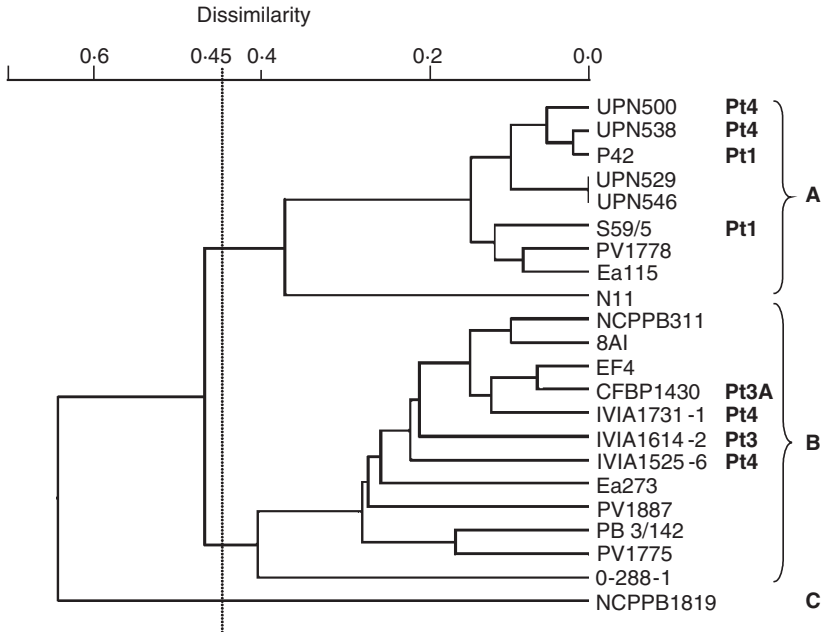

Fig. 3 Dendrogram of *Erwinia amylovora* strains based on UPGMA cluster analysis with the NTSYS-PC program using the combined AFLP fingerprints with four different primer combinations. The dendrogram was constructed using Jaccard's dissimilarity coefficients; the UPGMA clusters (45% cut off) are indicated with letters. Pt3, Pt3A and Pt4 indicate PFGE patterns as previously described (Zhang *et al.* 1998; Jock *et al.* 2002)

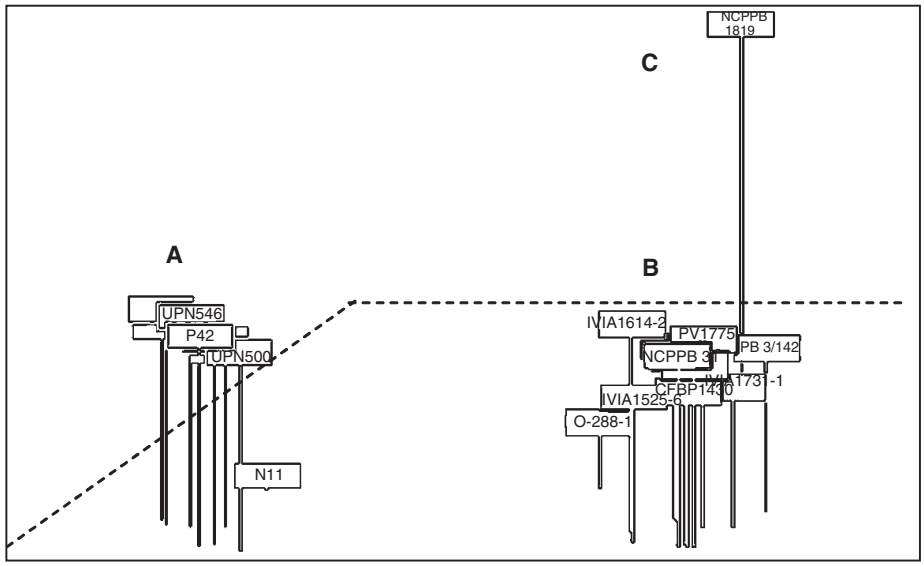

Fig. 4 Principal coordinate analysis (PCA) performed with *Erwinia amylovora* strains based on Euclidean similarity coefficients. PCA groups are indicated by capital letters, which also correspond to the UPGMA clustering. The first three dimensions explain 81·8% of the variation observed. Group A: UPN500; UPN529; UPN538; UPN546; P42; S59/5; N11; PV1778; Ea115; Group B: EF4, PB3/142, NCPPB311; 0-288-1; IVIA1614-2; 8AI; PV1887; PV1775; IVIA1525-6; IVIA1731-1; CFBP1430; Ea273; Group C: NCPPB1819

relation was detected between the strains grouped in cluster A and B, and the host, place or year of isolation.

Principal coordinate analysis

In addition to the cluster analysis, we performed a Principal Coordinate Analysis (PCA) as an statistical ordination method (Gower 1966). Pairwise squared Euclidean distances based on the band scoring values were calculated for the 22 *E. amylovora* strains in order to derive a double-centered distance matrix for factoring (Rohlf 1972). The plot of the first three coordinates, which explains 81·8% of the variation observed, is shown in Fig. 4. The results showed minor differences with those obtained by cluster analysis. Most of

the strains grouped closely together within each group and were positioned further apart between groups than in the dendrogram.

DISCUSSION

Our results clearly indicate that the AFLP technique can successfully be used to reveal substantial genetic variability among *E. amylovora* strains. This is significant because previously used molecular typing techniques, such as RAPD or rep-PCR fingerprinting, have proved to be of very limited value in establishing genealogies or phylogenetic relationships (Momol and Aldwinckle 2000). These techniques allowed the unequivocal differentiation of strains isolated from either Maloideae hosts, *Rubus* or Asian pear, but had a limited resolution power within the group of strains infecting Maloideae plants, such as the strains used in this work. Additionally, as AFLP analysis has a high resolution power that is comparable with DNA pairing studies (Clerc *et al.* 1998), this typing method could be used in the future to reveal patterns of dispersal by this pathogen. To our knowledge, there are no previous published reports on the application of AFLP in the analysis of *E. amylovora* populations.

In this study, four combinations of primers with one selective nucleotide were selected because they produced informative fingerprints that differentiated 21 of 22 *E. amylovora* strains tested. As expected, AFLP provided a significantly higher resolution than the rep-PCR analysis (compare Figs 1 and 3), because a larger number of bands were considered in the cluster analysis. In fact, most of the strains showed identical rep-PCR patterns and only the most divergent strain, NCPBB1819, clustered similarly in both dendrograms. As the polymorphic PCR bands that identified NCPPB1819 had phylogenetic significance, this highlights the potential value of certain PCR bands as sensitive and effective molecular markers for strain typing. Moreover, the quality of the differentiation of strains by AFLP is reflected in the highly significant correlation between the dissimilarity and the tree matrices from Cluster analysis and the high portion of explained variance in PCA.

Recently, PFGE analysis of genomic DNA after *Xba*I digestion was used to group *E. amylovora* strains from Maloideae hosts into six major pattern types (Pt1–Pt6) that offered clues to potential dispersal routes of the pathogen (Zhang and Geider 1997; Jock *et al.* 2002). We observed discrepancies between the genealogy derived from AFLP analysis and that obtained by PFGE. In particular, four strains from Northern Spain that shared the PFGE profile Pt4 (Zhang and Geider 1997; Jock *et al.* 2002) were separated in two clusters by AFLP analysis (Fig. 3), whereas strains showing different PFGE patterns (Pt3 and Pt4, or Pt1 and Pt4) clustered together in the AFLP dendrogram. Currently, it is difficult to resolve these discrepancies because the molecular basis of the observed PFGE polymorphisms are not known. The six PFGE patterns differ in the position of one or two bands from 50 to around 250 kb (Zhang and Geider 1997; Jock *et al.* 2002) and could be the result of a large variety of DNA changes (Tenover *et al.* 1995). If the generation of one or more of these patterns results from DNA changes that occur repetitively in the bacterial population, then the classification of strains by PFGE patterns would not necessarily correlate with their phylogenetic origin (Gürtler and Mayall 2001). Sequence analysis of six of the seven rRNA operons of *E. amylovora* has revealed sequence and length polymorphism in the intergenic transcribed spacers, some of which contain an *Xba*I restriction site (McGhee *et al.* 2002); further, the type of spacers and the number of *Xba*I sites were variable among different *E. amylovora* strains. Multigene families, such as the rRNA gene family, have a tendency towards becoming identical in DNA sequence by homologous recombination (Gürtler 1999; Gürtler and Mayall 2001), or concerted evolution, and there is evidence that recombination between copies of the spacers has occurred in the chromosomes of *E. amylovora* (McGhee *et al.* 2002). However, it is unknown how often these rearrangements would occur, or even if they would affect the PFGE patterns after *Xba*I digests. In this context, the PFGE patterns where shown to be stable after 16 transfers from single colony on LB agar plates and be independent of changes in different characteristics of the strains, such as levan production or amylovoran synthesis (Zhang and Geider 1997; Zhang *et al.* 1998). In contrast, AFLP revealed a large number of polymorphic bands that allowed grouping of the *E. amylovora* strains with a high degree of confidence (Figs 3 and 4). For instance, strains UPN500 and IVIA1731-1 (both Pt4) were differentiated by 20 AFLP bands; conversely, UPN500 was differentiated from P42 (Pt1) by only two AFLP bands. Nonetheless, we cannot discount the possibility that PFGE and AFLP reflect the differential evolution of different genome regions, although it would be necessary to investigate the ultimate causes of the PFGE polymorphisms in order to assess its usefulness in phylogenetic studies.

Grouping of strains in the AFLP dendrogram (Fig. 3) was not obviously correlated with host, place, or year of isolation. The lack of correspondence between genealogy and geographical origin is not surprising and agrees with the results obtained using biochemical, physiological, serological or genetic characteristics (Momol and Aldwinckle 2000; Brennan *et al.* 2002). McManus and Jones (1995) suggested that it is reasonable to expect a higher variability among strains from North America, which is the recognized centre of origin of this pathogen, than among strains from Europe, where the disease has been present only for the last 45 years (van der Zwet and Wells 1993). However, and taken into

account that we have used a different set and a limited number of *E. amylovora* strains compared with other studies, our results indicate a similar degree of divergence among the American and the European strains. This could be explained if we assume that a given set of European strains could have originated from a given strain from North America. Indeed, the presence of both European and American strains within AFLP clusters A and B strongly suggests that there have been multiple introductions of *E. amylovora* into Europe. Likewise, the separate grouping of Spanish strains in clusters A and B, even of those strains coming from the same province (e.g. UPN500 and IVIA1731-1), suggests the occurrence of multiple introductions into Spain. An alternative explanation is that only one or a few introductions occurred, but the plants were infected with multiple strains of the pathogen. To confirm the hypothesis of multiple introductions it would be necessary to analyse a significantly larger number of strains from different locations. On the basis of the success with other bacterial species (Janssen *et al.* 1996; Clerc *et al.* 1998; Restrepo *et al.* 1999; Rademaker *et al.* 2000), the AFLP technique should prove highly valuable in ecological, epidemiological and evolutionary studies aimed at investigating genetic diversity of *E. amylovora* worldwide.

ACKNOWLEDGEMENTS

We are grateful to D. Berra, M. Borruel, M.M. López and G. Sundin for providing *E. amylovora* strains, to I. Barrena for expert technical assistance and T. Williams for critical reading of the manuscript. This research was supported with grants AGF98-0402-CO3-O3 and AGL2001-1948-CO2-01, from the Spanish CICYT. AR was funded by a fellowship from the Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA).

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