Polymerase Chain Reaction Fingerprinting of *Erwinia amylovora* has a Limited Phylogenetic Value but Allows the Design of Highly Specific Molecular Markers

Arantza Rico, M. Elena Führer, Amaya Ortiz-Barredo, and Jesús Murillo

First, second, and fourth authors: Laboratorio de Patología Vegetal, Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Spain; third author: NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Granja Modelo de Arkaute, Apartado 46, 01080 Vitoria, Spain.

Current address of first author: Department of Plant Sciences, University of Oxford, South Parks Road, OX1 3RB, Oxford, UK.

Accepted for publication 24 September 2007.

ABSTRACT

Rico, A., Führer, M. E., Ortiz-Barredo, A., and Murillo, J. 2008. Polymerase chain reaction fingerprinting of *Erwinia amylovora* has a limited phylogenetic value but allows the design of highly specific molecular markers. *Phytopathology* 98:260-269.

Erwinia amylovora, the causal agent of fire blight, is genetically very homogeneous, and current methodologies provide insufficient or contradictory information about the probable dispersal routes of the pathogen. With the final aim to obtain specific and reliable molecular markers for different lineages of the pathogen, we studied the molecular basis of rep-polymerase chain reaction (PCR) polymorphism using seven different arbitrary primers to fingerprint 93 *E. amylovora* strains from different countries, including Spain. Polymorphism was very low, and was displayed by only 11 *E. amylovora* strains, which produced 22 polymorphic bands. Five of

11 polymorphic bands cloned contained DNA that was present in more than 85% of the strains, whereas six bands were due to DNA present exclusively in the strains producing the rep-PCR polymorphism. Also, five of the polymorphic bands were due to the possession of either the ubiquitous plasmid pEA29, of plasmid pEU30, which was exclusively found in strains from North America, or of a 35-kb cryptic plasmid, present only in 28 strains from Northern Spain. We designed primer pairs from several cloned polymorphic bands that allowed the specific identification of the strains producing the polymorphism. Our results indicate that rep-PCR is not adequate for constructing genealogies of *E. amylovora*, although the strategy illustrated here, as well as the designed primers, can be used effectively in epidemiological studies with this pathogen.

Additional keywords: *Maloideae*, native plasmids, *rpsU-dnaG*.

Fire blight is a destructive bacterial disease caused by the gram-negative bacterium *Erwinia amylovora*. The economically most important host species belong to the subfamily *Maloideae* of the *Rosaceae* family, which includes apple and pear trees (33). Strains of *E. amylovora* can also infect species of *Rubus*, such as raspberry and blackberry (46), although these strains have been only isolated in the United States and are genetically separable from the strains isolated from *Maloideae* hosts (20,30,32,35). Fire blight was first reported in the East coast of the United States in the 18th century and in Europe around 1958, spreading later to most of the continent and most of the Mediterranean countries (5). The first report of fire blight in Spain was in 1995 in a cider-apple orchard in Guipúzcoa near the French border (25) and, in the following years, outbreaks were reported in several locations through the Northeast of Spain and in two nurseries of Central Spain (26). However, the eradication measures were highly successful and the pathogen did not spread further, being virtually eliminated from the affected areas (10).

E. amylovora is considered a genetically homogeneous species as confirmed by studies ranging from host range, morphology, serology, and biochemical profiles (12,16,20,33). The genetic diversity of *E. amylovora* has been explored by polymerase chain reaction (PCR) fingerprinting (29,32,35), pulse field gel electrophoresis (PFGE) (9,19,52), phylogenetic analysis of conserved sequences of DNA (17,21,28), and amplified fragment length

polymorphism (AFLP) (9,41). Currently, AFLP is by far the most discriminating technique, although both AFLP and PFGE are time consuming techniques that are not suitable for the analysis of large numbers of strains. An additional complication is the well established lack of correlation between strain groupings obtained from AFLP and PFGE data (9,41), which makes it difficult to reach convincing conclusions from epidemiological studies using these techniques.

Further works attempted to find additional tools to uncover the genetic variability of *Maloideae* strains; in particular, the availability of rapid and sensitive techniques that allow the analysis of large numbers of strains is highly desirable to evaluate the dispersal routes of the pathogen. The number of short sequence repeats contained in the ubiquitous plasmid pEA29 (21) is highly variable among strains, even if they were isolated in the same orchard; however, the number of repeats within a strain appeared to change under stresses induced experimentally, which limited the use of this feature for the classification of strains (3,18). The analysis of a group of *Maloideae* strains from diverse origins by AFLP (41) revealed higher genetic variability among *E. amylovora* strains from different locations than previously used methods. The variability observed among the European strains suggested that multiple introductions of fire blight within the continent could have occurred, although the ultimate origin of the foci still remains obscure due to the lack of appropriate epidemiological markers to trace pathogen dispersal. The ability of AFLP to detect polymorphisms was compared to rep-PCR (41), which is simpler and faster for the analysis of large numbers of strains, although it is less discriminative. However, some of the rep-PCR polymorphic bands could differentiate an American strain that

Corresponding author: J. Murillo; E-mail address: jesus.murillo@unavarra.es

doi:10.1094/PHYTO-98-3-0260

© 2008 The American Phytopathological Society

was also divergent in the AFLP analyses. Thus, this type of polymorphism could have epidemiological significance and could be used to design highly specific molecular markers for strain typing, as shown previously with other bacterial pathogens (27,39,40,50), or for epidemiological studies to trace the dispersal from a given focus. Besides, PCR amplification is faster to perform than either AFLP or PFGE, and allows the analysis of a large number of bacteria.

In this study, we used rep-PCR to examine the genetic diversity of 93 *E. amylovora* strains from various hosts, years, and origins of isolation. We investigated the molecular basis of the polymorphism generated by seven arbitrary primers, and the ability of this fingerprinting technique to generate highly specific molecular markers for *E. amylovora*. The polymorphism found was limited, and in half of the cases, was due to the existence of specific DNA in individual strains, either in plasmids or in the chromosome. Our results suggest that, although this polymorphism has a limited

application to the generation of genealogies, the majority of the polymorphic bands could be used to generate highly specific molecular markers. These markers could be useful in epidemiological studies, to trace the origin and routes of dispersal of a given outbreak.

MATERIALS AND METHODS

Strains and growth conditions. The 93 *E. amylovora* strains used in this work, and their characteristics, are listed in Table 1. All the strains produced the expected 844-nt amplicon after PCR amplification with primers AJ75 and AJ76 (29) directed to a conserved region of the ubiquitous 29-kb plasmid pEA29 of *E. amylovora*. The exception was strain IVIA1614-2 that lacks this plasmid (24).

Strains *E. coli* CECT831 (Colección Española de Cultivos Tipo, Spain) and *P. syringae* pv. *phaseolicola* 1449B (49) were

TABLE 1. Country, host, and year of isolation of *Erwinia amylovora* strains^a

Country	Name	Host and year of isolation ^b	Country	Name	Host and year of isolation ^b
Bulgary	EaFG2	<i>Pyrus</i> sp., nk		IVIA1898-11	<i>Pyrus</i> sp., Guadalajara, 1998
Canada	CFBP3050	<i>Malus</i> sp., 1980		IVIA1898-18	<i>Malus</i> sp., Guadalajara, 1998
	NCPBP311	<i>Pyrus</i> sp., 1952		IVIA1899-19	<i>Pyrus</i> sp., Guadalajara, 1998
Cyprus	GCCM841	<i>Pyrus</i> sp., nk		IVIA1899-21	<i>Cydonia</i> sp., Guadalajara, 1998
Czech Republic	GCCM1634	<i>Sorbus</i> sp., nk		IVIA1909-3	<i>Pyrus</i> sp., Lérida, 1998
Egypt	INRA2279	nk, nk		IVIA1924-4	<i>Pyracantha</i> sp., Huesca, 1998
	NCPBP1734	<i>Pyrus</i> sp., 1965		IVIA1951-2	<i>Pyracantha</i> sp., Huesca, 1998
France	CFBP1430	<i>Crataegus</i> sp., France, 1972		IVIA1951-5	<i>Cotoneaster</i> sp., Huesca, 1998
	CFBP2314	<i>Malus</i> sp., nk		IVIA1951-6	<i>Sorbus</i> sp., Huesca, 1998
	INRA2543	<i>Malus</i> sp., nk		IVIA1951-8	<i>Pyracantha</i> sp., Huesca, 1998
	INRA2553	nk, nk		IVIA1961-5	<i>Pyrus</i> sp., Guipúzcoa, 1998
	INRA2561	nk, nk		IVIA1961-16	<i>Pyrus</i> sp., Guipúzcoa, 1998
	INRA2576	nk, nk		IVIA1981-1	<i>Crataegus</i> sp., Segovia, 1996
	INRA2581	<i>Malus</i> sp., 1992		IVIA1984-2	<i>Crataegus</i> sp., Segovia, 1996
	INRA2597	<i>Malus</i> sp., nk		IVIA1985	<i>Pyracantha</i> sp., Madrid, 1998
	LNPV1099	<i>Pyracantha</i> sp., uk		UPN500	<i>Pyrus</i> sp., Guipúzcoa, 1998
	LNPV1626	<i>Pyrus</i> sp., nk		UPN511	<i>Malus</i> sp., Guipúzcoa, 1998
	LNPV1888	<i>Cotoneaster</i> sp., nk		UPN525	<i>Pyracantha</i> sp., Navarre, 1997
	LNPV2026	<i>Cotoneaster</i> sp., nk		UPN526	<i>Pyrus</i> sp., Navarre, 1997
	LNPV2030	<i>Pyracantha</i> sp., nk		UPN528	<i>Malus</i> sp., Navarre, 1997
	PV1775	<i>Cotoneaster</i> sp., nk		UPN530	<i>Pyrus</i> sp., Navarre, 1997
	PV1778	<i>Cotoneaster</i> sp., nk		UPN537	<i>Malus</i> sp., Guipúzcoa, 1998
	PV1887	<i>Cotoneaster</i> sp., nk		UPN538	<i>Cotoneaster</i> sp., Guipúzcoa, 1998
Greece	BPIC843	<i>Pyrus</i> sp., 1984		UPN540	<i>Pyrus</i> sp., Navarre, 1998
	BPIC909	<i>Pyrus</i> sp., 1985		UPN545	<i>Malus</i> sp., Navarre, 1998
	BPIC917	<i>Pyrus</i> sp., 1985		UPN546	<i>Cydonia</i> sp., Navarre, 1998
	BPIC932	<i>Malus</i> sp., 1986		UPN547	<i>Pyracantha</i> sp., Navarre, 1998
	BPIC1624	<i>Pyrus</i> sp., 1990		UPN610	<i>Pyracantha</i> sp., Navarre, 1998
Germany	CFBP3056	<i>Cotoneaster</i> sp., nk		UPN564	<i>Cotoneaster</i> sp., Navarre, 1998
	Ea115	<i>Malus</i> sp., 1989	The Netherlands	NCPBP3159	<i>Malus</i> sp., 1980
Ireland	SL2156	<i>Cotoneaster</i> sp., nk	Turkey	NCPBP3548	<i>Eriobotrya</i> sp., 1987
	SL2157	<i>Cotoneaster</i> sp., 1993	United Kingdom	Ea3042	<i>Pyrus</i> sp., nk
	SL2158	<i>Sorbus</i> sp., 1993		NCPBP595	<i>Pyrus</i> sp., 1958
	SL2159	<i>Sorbus</i> sp., 1993		NCPBP683	<i>Pyrus</i> sp., 1959
	SL2160	<i>Sorbus</i> sp., Ireland, 1993		Ea273	<i>Malus</i> sp., 1971
	SL2162	<i>Sorbus</i> sp., 1993	USA	NCPBP1819	<i>Crataegus</i> sp., 1966
	SL2164	<i>Sorbus</i> sp., 1993		NCPBP2292	<i>Rubus idaeus</i> , 1970
Italy	OMP-BO 1185	<i>Malus</i> sp., nk		NCPBP2791	<i>Pyrus communis</i> , 1975
Israel	CFBP3098	<i>Malus</i> sp., 1987		IVIA1626	<i>Malus</i> sp., Navarre, 1996
New Zealand	NCPBP2080	<i>Pyrus</i> sp., 1968		IVIA1626-6	<i>Malus</i> sp., Navarre, 1997
Sweden	CFBP2582	<i>Pyrus</i> sp., nk		IVIA1731-1	<i>Pyrus</i> sp., Guipúzcoa, 1997
Spain	IVIA1509	<i>Malus</i> sp., Guipúzcoa, nk		IVIA1767-3	<i>Malus</i> sp., Navarre, 1997
	IVIA1525-1	<i>Cotoneaster</i> sp., Guipúzcoa, 1996		IVIA1739-1	<i>Malus</i> sp., Gerona, 1997
	IVIA1525-6	<i>Cotoneaster</i> sp., Guipúzcoa, 1996		IVIA1777-1	<i>Pyracantha</i> sp., Alava, 1997
	IVIA1578-3	<i>Crataegus</i> sp., Segovia, 1996		IVIA1892-1	<i>Pyrus</i> sp., Guadalajara, 1998
	IVIA1603	<i>Cotoneaster</i> sp., Segovia, 1996		IVIA1898-4	<i>Cotoneaster</i> sp., Guadalajara, 1998
	IVIA1614-2	<i>Pyracantha</i> sp., Segovia, nk			

^a All strains were obtained from M. M. López (IVIA), except UPN strains, that were obtained from D. Berra (Laboratorio Agrario, Guipúzcoa, Spain) and M. Borruel (Laboratorio Agrario, Navarre, Spain). BPIC, Benaki Phytopathological Institute Collections of Fungi and Bacteria, Greece; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; GCCM, Greek Coordinated Collections of Microorganisms, Greece; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; NCPBP, National Collection of Plant Pathogenic Bacteria, York, UK; PV, Protection des Végétaux, Angers, France; UPN, Universidad Pública de Navarra, Pamplona, Spain.

^b nk: not known.

used as negative controls in dot blot experiments. Strain *E. coli* 39R861 (36) and reference *E. amylovora* strain CFBP1430 (38) were used as molecular size markers and positive control, respectively, in the native plasmid gel preparations.

All strains were propagated on Luria Bertani medium (LB) (44) at 28°C and maintained at -80°C in LB plus 20% glycerol for long-term storage.

PCR analyses. PCR fingerprinting was performed using primers ERIC1R-ERIC2, BOXA1R (30), a single 20-bp oligonucleotide primer complementary to the IS50 portion of Tn5 (primer IS50) (48), CAG5 (42), GTG5 (51), and the sequencing primers M13 forward (FOR, 5'-CCCAGTCACGACGTTGTA-AAACG-3') and M13 reverse (REV, 5'-AGCGGATAACAATTT-CACACAGG-3') (8). PCR reactions were carried out in a volume of 25 µl containing 5 µl of a bacterial suspension. For primers FOR, REV, CAG5, and GTG5, 1× PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 10 pmol primer, and 1 U of *Taq* DNA polymerase (Biotaq-Bioline, UK) were used. The PCR reactions consisted of a denaturation step of 10 min at 94°C, followed by 2 cycles at 94°C for 1 min, 40°C for 5 min and 72°C for 5 min and by 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 primers ERIC, BOXA1R, and IS50, the PCR reactions were performed under the conditions described for each primer.

DNA fragments from gene *repA* from plasmid pEA29 and the chromosomal *rpsU-dnaG* operon from *E. amylovora* Ea273 were amplified using the primers indicated in Table 2. Amplifications of DNA fragments specific to different *E. amylovora* strains analyzed in this study were performed with primer pairs UNE1-F/R, UNE7-F/R, UNE8-F/R, and UNE9-F/R (Table 2). Reactions were carried out in a final volume of 25 µl containing 5 µl of a bacterial suspension, 1× PCR buffer, 1.5 mM MgCl₂, 0.15 mM each dNTPs, 20 pmol of each specific primer, and 1 U of *Taq* DNA polymerase (Biotaq, Bioline, UK). Conditions were a denaturation step of 5 min at 94°C followed by 26 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 7 min.

Amplifications were carried out using a RoboCycler Gradient Cycler (Stratagene) and products were separated by electrophoresis on 1.5% agarose gels and visualized under UV light after ethidium bromide staining (44). Each PCR was performed at least twice in separate experiments and, for PCR fingerprinting, only reproducible bands between 0.1 and 2 kb were considered.

Molecular techniques. Native plasmids were isolated using a NucleoBond Plasmid Purification Maxi kit (Clontech Laboratories, Palo Alto, CA) and separated uncut on 0.5% agarose gels using 1× TAE buffer during 4 to 5 h at 90 V at room temperature. For Southern and dot blot hybridizations, genomic DNA was isolated from single colonies grown overnight in LB using a DNA

extraction kit (Puregene, Gentra Systems). Genomic DNA treated with restriction enzymes and uncut plasmid DNA separated on agarose gels were transferred to positively charged nylon membranes for Southern hybridizations (2,44). For cloning, PCR bands were excised from the gels and purified using the GFX-PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech), and ligated to pGEM-T Easy Vector (Promega). Cloned DNA fragments to be used as probes were amplified by PCR using insert-specific primers. The resulting amplicons were separated on agarose gels, purified from the gel and labeled by random priming with [α -³²P]dCTP or with digoxigenin (Roche, Basel, CH). Cloned polymorphic bands were partially or completely sequenced using MWG-Biotech Sequencing services. The corresponding nucleotide sequences, excluding the sequences derived from the amplification primers, were compared against the nucleotide sequence databases using BLAST algorithms (1). Primers were designed using the Primer3 software (43) and were synthesized by the Sigma-Genosys Oligo Synthesis service. Sequence alignments were performed using Multalin (7). Nucleotide sequences were deposited in the nucleotide sequence database NCBI under the following accession numbers: DQ660310 to DQ660312 for bands E2, P1, and I7; and EF679781 to EF679786 for bands E4, E5, R3, R4, E3, and I4, respectively.

RESULTS

Identification and cloning of polymorphic bands. Primer pair ERIC1R-ERIC2 and primer IS50 were used to fingerprint 93 *E. amylovora* strains from various hosts and origins with the aim to identify and clone reproducible polymorphic bands. The band patterns obtained with these primers were highly reproducible and, in general, showed little variation among the strains examined. The ERIC primers produced a total of 13 bands, of which eight were polymorphic and defined five different patterns (Fig. 1, Table 3); pattern A was the most common, appearing in 86 strains. Primer IS50 yielded five polymorphic bands of a total of 11 bands, and five patterns. Again, the most frequent pattern was shared by the other 89 strains.

In order to explore more variability, we tested primers BOXA1R, CAG5, GTG5, FOR, and REV with 54 of 93 *E. amylovora* strains. We did not detect reproducible polymorphisms with primers BOXA1R, GTG5, and FOR. Primer REV produced eight polymorphic bands from a total of 11 reproducible bands, and four patterns. The most frequent pattern was shared by 51 strains (Fig. 1, Table 3). The amplification with primer CAG5 produced a total of nine bands, of which two were polymorphic and defined three different patterns (Fig. 1, Table 3).

In general, the *E. amylovora* strains were highly homogeneous and all the observed polymorphisms were displayed by only 11 of

TABLE 2. Primers designed in this work for strain identification

Primer pair	Sequence (5'–3')	Fragments amplified
repA–F repA–R	GATGAAATGCGTTTGCTGAA CAGTCCCGATATTGCTCCAT	772-bp fragment from gene <i>repA</i> of pEA29
rpsU–L DnaG–R	GAAAAACCGACTACCGA TCGTTTGTACACGATACC	0.7- to 1.1-kb fragment containing partial coding region of <i>rpsU</i> and <i>dnaG</i> and the intergenic DNA
R4273L R4273R	TTTAGCTGCCTCTTCGCTGT TGGCTGACAGGGGATAACAT	1-kb fragment containing the amplification sites for band R4
UNE1–F UNE1–R	GAGCGCGTTTTTCGGCATGC TGAGCGGTAGATAACATCGCT	0.2-kb fragment specific of 28 strains from Navarre, Spain; primers internal to band E2
UNE7–F UNE7–R	TGCCTTTGAGTGCGTATT TCATGGAAATATGACAAC	0.15-kb fragment specific to strain UPN526 from Navarre, Spain; primers internal to band I7
UNE8–F UNE8–R	GGATATTACAGCCAGCTCAGA CAGGTCGTTACTTTATCCTG	0.9-kb fragment specific to strain NCPB1819, from USA; primers internal to band R3
UNE9–F UNE9–R	GAGCAACGCAAAAGCACATG AGCAACAGCCGTAGCGTTATC	0.4- kb fragment specific of 28 strains from Navarre, Spain; primers internal to band P1
UNE11–F UNE11–R	GCGGGCAGGAAATTTTGACG TTCACCCGTTTCATCGATGGTG	0.2-kb fragment specific to strain SL2162; primers internal to band E3

the strains (Table 4). Strain NCPPB2292, the only one isolated from the non-*Maloideae* host *Rubus* sp., showed the highest number of polymorphisms (11 polymorphic bands), as expected. Conversely, strains isolated from *Maloideae* showed only one to three polymorphic bands.

Analysis of polymorphic bands representing common DNA. Five of 11 polymorphic bands examined (E4, E5, I6, R3, and R4; Fig. 1, Table 5) contained DNA that was present in more than 85% of the 93 strains examined. Four of these bands (E4, E5, R3, and R4) hybridized to all the strains in dot blot experiments, suggesting that they were differentially amplified due to changes in the primer binding sites or to changes in the size or the organization of the amplified sequences.

The ERIC amplification profile of strain LNPV1099 contained band E5 (741 nts) but not band E4 (1,233 nts), which was present

TABLE 3. Number of patterns and polymorphic bands produced by polymerase chain reaction fingerprinting of *Erwinia amylovora* strains with different primers^a

Primers	Band patterns	No. of bands	
		Total	Polymorphic
ERIC	5	13	8
ISS0	5	11	5
REV	4	11	8
CAG5	3	9	2
BOXA1R	1	9	0
GTG5	1	4	0
FOR	1	4	0

^a Ninety-three strains were analyzed with the enterobacterial repetitive intergenic consensus (ERIC) primer pair and primer ISS0, and 54 strains with the remaining primers.

Fig. 1. PCR fingerprinting patterns of *Erwinia amylovora* strains with the primers indicated. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light. The pattern type for each primer condition and the number of *E. amylovora* strains displaying each pattern are indicated above each lane. Arrowheads indicate polymorphic bands, with their designation; bands that were cloned and characterized are underlined. Due to its differential intensity, we assumed that band I2 was polymorphic, although we were unable to clearly separate it from a putative co-migrating band. M, 100-bp marker; the size or relevant marker bands are indicated in kilobases to the left.

in the profile of the remaining strains examined. The complete nucleotide sequence of E5 was identical to the 3' end of band E4 (Fig. 2A). In a tblastx homology search, the sequences of both bands showed significant homology to *dnaG* of *E. coli* (accession no. J01687), which encodes the primase protein and is essential for chromosomal replication. Primers were then designed from the *E. coli rpsU* gene, which precedes *dnaG* in this bacterium, and from the *E. amylovora dnaG* sequence in order to amplify the DNA containing the left putative ERIC binding sites that allowed the amplification of bands E5 and E4 (Fig. 2A). These primers amplified bands of 1 kb in 91 of 93 strains of *E. amylovora* examined, 1.1 kb in strain NCPPB1819 and 0.75 kb in strain LNPV1099 (Fig. 2B), indicating that the *rpsU-dnaG* region is variable. The analysis of the nucleotide sequence of the amplicons from strains SL2162, NCPPB1819, and LNPV1099 showed that this gene region has the same overall organization as *E. coli*, and that the coding regions of *rpsU* (64 nts examined) and *dnaG* (1,136 nts examined) were well conserved. However, the intergenic sequence between *rpsU* and *dnaG* was different in size in *E. coli* (111 nts) and in *E. amylovora* strains SL2162 (418 nts), NCPPB1819 (543 nts), and LNPV1099 (168 nts). The 111 nts sequence present in *E. coli* was poorly conserved in the *E. amy-*

lovora strains (57% identity), except for the *rpsU* terminator signal that was identical. Strain SL2162 contained an additional sequence of 293 nts that included two tandem imperfect 125 nt repeats, differing in 2 nts, followed head to tail by a sequence that spanned the first 43 nts of the 125 nt repeats (Fig. 2C). The two putative ERIC elements in *E. amylovora* strain SL2162 contained a sequence (L4; Fig. 2C) that showed only two mismatches to primer ERIC2; the rightmost of these two ERIC2-like sequences served as substrate for the amplification of the cloned E4 band. In strains NCPPB1819 and LNPV1099, the intergenic region was almost identical to that of strain SL2162, except that strain NCPPB1819 contained a third 125 nt repeat. While the first 125 nt repeat of NCPPB1819 was identical to the first repeat in strain SL2162 (repeat a, Fig. 2C), the second and third repeats were identical between them and showed only a nucleotide changed compared to repeat a (Fig. 2D). In strain LNPV1099 only the 43 nt repeat was present (Fig. 2B). This suggests that the 125 nt repeats were deleted in LNPV1099, resulting in the concomitant loss of the annealing sites for ERIC2; consequently, the amplification of the cloned band E5 from strain LNPV1099 took place through the annealing of the 3' end of primer ERIC2 to a 14 nt sequence that showed three internal mismatches compared to this

TABLE 4. *Erwinia amylovora* strains showing specific polymorphic bands by polymerase chain reaction (PCR) fingerprinting with different primers

Primers used	Strains	PCR bands ^a	
		Additional	Absent
ERIC, IS50	UPN546, UPN547, UPN564 ^b	<u>E2</u>	
	UPN526 ^b	<u>E2</u> , I2, I7	
	LNPV1099	<u>E5</u>	<u>E4</u>
	IVIA1614-2		<u>I6</u>
ERIC, IS50, REV, CAG5	NCPBP311	<u>I4</u>	
	NCPBP1819	<u>R3</u>	<u>R4</u>
	NCPBP2292	E6, E7, E8, I3, P3, R1, R2, R6	E9, R5, R9
	SL2162	<u>E3</u> , <u>R8</u>	
	UPN610	<u>E2</u> , <u>P1</u>	

^a The letters indicate the primers that produced the polymorphic bands. E: ERIC, I: IS50, R: REV, P: CAG5. The underlined bands were cloned and further characterized. Due to its differential intensity, we assumed that band I2 is polymorphic, although we were unable to clearly separate it from a putative co-migrating band.

^b Although they were not originally tested with primer CAG5, these four strains also produced polymorphic band P1 upon rep-PCR amplification with this primer.

TABLE 5. Characteristics of the studied polymerase chain reaction (PCR) polymorphic bands

Band	Size (bp)	PCR information	Genomic location	Homology (accession no.) ^a
DNA present in more than 85% of the strains				
E4	1,233	Absent from LNPV1099	Chromosome	<i>E. coli dnaG</i> (J01687)
E5	741	Present in LNPV1099	Chromosome	<i>E. coli dnaG</i> (J01687)
I6	238	Absent in IVIA1614-2	Plasmid	<i>betT</i> , <i>E. amylovora</i> pEA29 plasmid (AF264948)
R3	910	Present in NCPBP1819	Chromosome	Putative ORF from the CP4-6 prophage from <i>E. coli</i> (NP_414786); IS1637, from <i>Yersinia enterocolitica</i> (AF102990)
R4	797	Absent from NCPBP1819	Chromosome	Thiosulfate binding protein precursor, <i>Salmonella enterica</i> (CP000026)
DNA present in less than 85% of the strains				
E2	225	Present in 28 <i>E. amylovora</i> Spanish strains ^b	Plasmid	Conjugative transfer protein in pEU30 of <i>E. amylovora</i> strain UTRJ2 (NP_943280.1)
E3	250	Present in SL2162	Chromosome	Membrane protease subunits in <i>E. coli</i> , stomatin/prohibitin homologs (ZP_00730310.1)
I4	638	Present in NCPBP311	Plasmid	ORF25 in plasmid pEU30 from <i>E. amylovora</i> strain UTRJ2 (AY422215.1)
I7	149	Present in UPN526	Plasmid	<i>pilN</i> , located in megaplasmid of a STEC <i>E. coli</i> strain (AAL05518.1)
P1	457	Present in 28 <i>E. amylovora</i> Spanish strains ^b	Plasmid	Hypothetical protein in <i>Y. pseudotuberculosis</i> (YpseI_02003930)
R8	ca. 2.6 kb	Present in SL2162	Chromosome	Predicted membrane protein in <i>Y. mollaretii</i> of unknown function (ZP_00826271)

^a Homology searches were performed in GenBank using the blastx algorithm. None: no significant homologies found.

^b The strains containing DNA homologous to bands E2, I7 and P1, and producing the expected amplicons with primer pairs UNE1-F/R and UNE9-F/R are (all UPN strains) 526, 529, 531, 532, 534, 541, 542, 543, 546, 547, 550, 551, 552, 557, 560, 562, 563, 564, 567, 578, 579, 580, 581, 583, 594, 609, 610, and 611.

primer (L5, Fig. 2C). This putative binding site is located within the *dnaG* coding region and 466 nts downstream of L4.

Band I6 (238 bp) was absent from the IS50-PCR profile of strain IVIA1614-2 (Table 4) and did not hybridize to this strain in dot blot assays (data not shown). The sequence flanking the IS50 primer sites of band I6 was identical to a fragment of gene *betT* (accession no. AF164948), which is located in the ubiquitous plasmid pEA29 and codes for a high-affinity choline transport protein. Strain IVIA1614-2 does not contain plasmid pEA29 (24) and, as expected, this strain also failed to produce the specific amplicons using primer pair AJ75-AJ76 and a primer pair for gene *repA* (Table 2), coding for the putative replication protein of pEA29 (data not shown). Therefore, the absence of band I6 in the IS50-PCR profile of IVIA1614-2 is due to the lack of the complete pEA29.

Band R3 (910 bp) was specific to strain NCPPB1819 (Table 4). In Southern hybridizations of genomic DNA, this band hybridized

to a 12-kb *EcoRI* fragment from strain NCPPB1819 and to a 10-kb *EcoRI* fragment for the remaining *Maloideae* strains. Strain NCPPB2292, isolated from *Rubus* sp., showed a distinct hybridization pattern to band R3, consisting of three *EcoRI* strong hybridization bands of 10, 8, and 1.5 kb (data not shown). A blastn comparison showed that a region spanning nucleotides 1 to 114 showed 99% identity with internal sequences of IS1637, from plasmid pYVE227 from *Yersinia enterocolitica* (accession no. AF102990) whereas nucleotides 281 to 862 showed homology (63% aa identity) in a blastx search with hypothetical protein YafZ, a conserved protein from the CP4-6 prophage, from *E. coli* K12 (accession no. NP_414786). Primers UNE8-F/R, bordering internally band R3 in its entirety, produced a strong amplification band with strain NCPPB1819 and weak nonspecific amplifications with the remaining strains tested (data not shown); these unspecific bands disappeared when the annealing temperature of the primers was increased to 60°C. Primer UNE8-F is directed to

Fig. 2. A polymorphism in the ERIC-PCR fingerprint was due to changes in repetitive sequences in the *rpsU-dnaG* intergenic region of *Erwinia amylovora*. **A**, Organization of the *rpsU-dnaG* region of *Erwinia amylovora*. The relative location and size of amplicons E4 and E5 are indicated with thick lines, and the position of the primers used to amplify and sequence the left annealing sites of primer ERIC2 (*rpsU*-L and *dnaG*-R) are indicated with arrows. **B**, Amplification of the intergenic region between *rpsU* and *dnaG* using primers *rpsU*-L and *dnaG*-R. Strains of *E. amylovora* are SL2162, lane 1 (same pattern as other 90 strains); NCPPB1819, lane 2; LNPV 1099, lane 3; 100-bp molecular weight marker, lane M. **C**, Organization of the intergenic region between *rpsU* and *dnaG* in different strains of *E. amylovora*. The grey boxes indicate coding regions, and the location of the truncated 43 nt and the complete 125 nt ERIC repeats is indicated with arrows. L4 and L5 are the sites of annealing of primer ERIC2 during amplification of bands E4 and E5, respectively; sites L4 in parenthesis are potential sites of annealing. The sequences of L4 and L5 are compared to that of the ERIC2 primer, with mismatches in lowercase. **D**, Partial sequence of the 125 nt repeats and sequence of the 43 nt repeat. Letters a, b, and c refer to the type of repeat, as shown in panel C, with mismatches shown in boxes.

the end of IS1637, whereas UNE8-R binds to the homolog of *yafZ*, which results in the detection of a particular gene organization and the high specificity of this primer pair. Band R3, therefore, reflects a particular organization of genomic DNA that is in one or few copies and can successfully be used for accurate strain identification.

The sequence of band R4 (797 bp), absent from the REV-PCR profile of strain NCPPB1819, showed significant homology to the preceding sequences and 5' end of *cysP*, coding for a thiosulfate binding protein precursor from *Salmonella enterica* CT18 (accession no. CP000026) and was collinear with the draft genome of *E. amylovora* Ea273. To determine if the lack of amplification of band R4 in strain NCPPB1819 was due to nucleotide changes around the amplified sequence we designed primers R4273L and R4273R from the sequence of *E. amylovora* Ea273 that bordered the sequence homologous to that of band R4 (Table 2). These primers directed the amplification of a strong 1,010 nt band from strains NCPPB1819, which lacked band R4, and from Ea273 and CFBP1430, which contained band R4 in their REV-PCR profile. A sequence comparison of primer REV and the putative priming sites in strains Ea273 and NCPPB1819 showed seven mismatches in both strains in the forward priming site. In the reverse priming site the number of mismatches was 11 for strain Ea273 and 12 for strain NCPPB1819. These data suggest that the extra mismatch in strain NCPPB1819 did not allow the amplification of band R4 to occur in this particular strain.

Analysis of polymorphic bands representing specific DNA. Six of the cloned polymorphic bands (E2, E3, I4, I7, P1, and R8; Fig. 1 and Table 5) were strain-specific, because in dot blot assays they hybridized only with the strains that had produced the

corresponding band in the fingerprinting experiments or with less than 15% of the strains examined.

Band I4 (638 bp) was specific to strain NCPPB311, which was isolated from Canada, and showed hybridization only with this strain in dot blot and Southern hybridizations (data not shown). The band sequence was identical to internal sequences of ORF25 of plasmid pEU30 (accession no. NC_005247), which has been described only in 16 strains isolated from *Maloideae* hosts in the United States (11), suggesting that NCPPB311 might also contain pEU30.

Bands E2, I7, and P1 (225, 149, and 457 bp, respectively) hybridized with only five (UPN526, UPN546, UPN547, UPN564, and UPN610; Table 4) of 93 strains examined in dot blot hybridization experiments. We tested for the presence of these DNA fragments by PCR using internal primers from bands E2 (primers UNE1-F/R), I7 (primers UNE7-F/R), and P1 (primers UNE9-F/R) (Table 2) in our collection of 200 strains of *E. amylovora*, that includes 73 strains isolated from 1996 to 1998 in Navarra, Spain. From this screening, only 28 strains, isolated in Navarra, mostly from *Pyracantha* plants, produced the specific 0.2 and 0.45 kb amplification bands, respectively, with primer pairs UNE1 and UNE9 (data not shown). Although these 28 strains showed hybridization with band I7 in Southern blot experiments, only strain UPN526, which had produced the original band I7 with primers IS50, produced the expected 0.15 kb amplicon after PCR with primers UNE7-F/R (data not shown). This suggests the existence of sequence variability among the bacterial population containing DNA homologous to band I7. In a blastx search, band E2 showed a high similarity (57% aa identity) with the deduced sequence of *virB4* (accession no. NP_943280), a component of the type IV secretory pathway from plasmid pEU30 of *E. amylovora* (11). The sequence of band I7 showed homology (47% aa identity) to *pilN* from *E. coli* (accession no. AAL18829), coding for an outer membrane protein belonging to a type IV pilus (45). The 3' half of the sequence of band P1 showed homology (32% identity) to a hypothetical protein in *Y. pseudotuberculosis* (accession no. YpseI_02003930). It seems, therefore, that the existence of PCR polymorphic bands E2, I7, and P1 in these *E. amylovora* strains is probably due to horizontal DNA acquisition events.

Bands E3 and R8 (0.25 and ca. 2.6 kb) were specific for strain SL2162 in the ERIC and REV profile, respectively, and showed hybridization signals only with this strain in genomic DNA Southern blots. In a blastx search, band E3 showed homology (82% aa identity) to a membrane protease subunit, stomatin/prohibitin homolog, from the enteropathogenic *E. coli* E22 (accession no. ZP_00730310), but not to the draft genome of *E. amylovora* Ea273, suggesting that band E3 might represent DNA that has been horizontally acquired. PCR amplification using a pair of primers internal to E3 (UNE11-F/R) produced a strong 0.25-kb band with strain SL2162, which was isolated from *Sorbus* in Ireland, but no amplification products were obtained with the remaining 92 strains tested (data not shown). The sequence of one of the ends of band R8 showed homology (45% aa identity) in a blastx search with a predicted membrane protein of unknown function in *Yersinia mollaretii* (accession no. ZP_00826271). The sequence and distribution of band R8 was not further analyzed.

Genomic location of the highly specific polymorphic DNA.

The profiles of strain CFBP1430 and the eleven strains that showed polymorphisms contained at least one native plasmid (Fig. 3A), and all of them, with the exception of strain IVIA1614-2, contained a 29-kb plasmid that showed hybridization to a probe specific to the replicase gene, *repA*, of the ubiquitous plasmid pEA29 (Fig. 3B).

Probes specific to bands E3 and R8, both of which were specific of strain SL2162, did not hybridize to any of the native plasmids (data not shown), indicating that the DNA that originated from them is located in the chromosome. Conversely, the remaining four bands hybridized to plasmid DNA, suggesting a

Fig. 3. Genomic location of the DNA associated with some of the cloned polymorphic bands. **A**, Native plasmid profiles of *Erwinia amylovora* strains. Plasmids were isolated using a NucleoBond Plasmid Purification maxi kit and separated uncut by electrophoresis on a 0.5% agarose gel in 1x TAE. This and similar gels were blotted onto nylon and hybridized to probes labeled with digoxigenin corresponding to the coding sequence of the **B**, replication protein RepA from pEA29, and **C**, polymorphic bands I4, E2, I7, and **D**, P1, since these last three bands produced similar hybridization patterns, only a representative autoradiogram is shown. The arrowhead and asterisk in panel A indicate, respectively, the plasmids that hybridize to probes I4 and to the three probes E2, I7, and P1. Strains of *E. amylovora*: lanes 1, CFBP1430; 2, UPN500; 3, UPN526; 4, UPN546; 5, UPN547; 6, UPN610; 7, NCPPB311; 8, LNPV1099; 9, IVIA1614-2; 10, SL2162; 11, NCPPB1819; lane M, *E. coli* strain 39R861, used as MW marker. Sizes are indicated in kilobases. lpc: linear plasmids and chromosome.

possible horizontal acquisition. One of these, band I4, hybridized exclusively to a plasmid slightly larger than pEA29 present in strain NCPPB311 (Fig. 3C), which originally produced band I4. The size of this plasmid is similar to that of pEU30 (30 kb), which was described to be present in 16 strains from the United States (11). Since strain NCPPB311 was isolated in Canada, and the sequence of band I4 was identical to a gene from pEU30, it is likely that the plasmid hybridizing to band I4 is indeed pEU30. Although four strains from Spain also contained a plasmid of approximately 30 kb (Fig. 3C, lanes 3 to 6), it did not hybridize to I4, indicating that it might be a plasmid other than pEU30.

Bands E2, I7, and P1 produced strong hybridization signals with an approximately 35-kb plasmid that was only present in strains UPN526, UPN546, UPN547, and UPN610 among the 12 strains examined and, additionally, to a 29-kb plasmid band that co-migrated with pEA29 in strain NCPPB1819 (Fig. 3D). Therefore, the amplification of polymorphic bands E2, I7, and P1 is probably due to the presence in the bacterium of a single 35-kb plasmid, indicating that a single molecular event (i.e., the acquisition of a plasmid) can lead to significant changes in the rep-PCR profile.

DISCUSSION

The generation and comparison of PCR fingerprints by amplification of arbitrary or repetitive sequences has been widely used with bacteria for phylogenetic studies and for strain typing, especially in epidemiological studies with animal and plant pathogens (13,14,27). Additionally, cloned polymorphic bands have also been used extensively to differentiate bacterial species or populations (2,27,39), in many cases with the final aim to trace the dispersion pattern of a given strain in the environment. As with many other plant pathogens, PCR fingerprinting has also been applied to construct genealogies and to propose patterns of dispersal of *E. amylovora* (6,29,34,35). However, these applications have been hampered by the low diversity exposed by different PCR methods and by a general lack of knowledge to explain this diversity. In this work, we used diverse rep-PCR techniques to show the molecular basis behind the occurrence of PCR polymorphism in *E. amylovora* and, with this information we were able to design for the first time highly specific molecular markers that can be applied in epidemiological studies.

The use of seven different primer sets to fingerprint a collection of 93 *E. amylovora* strains from different countries revealed little polymorphism, as expected from previous results (6,9,29,34,35,41). Four primer sets produced a number of polymorphic bands, which varied between two with primer CAG5 to eight with the ERIC primer pair or the REV primer (Table 3). Nevertheless, 82 of the strains examined produced identical fingerprints for all the tested conditions, and only the remaining 11 strains showed variations with respect to the more frequent profiles (Table 4). This figure is probably an overestimation of the diversity, because four strains that showed identical polymorphisms (UPN546, UPN547, UPN564, and UPN610; Table 4) were isolated in the province of Navarre the same year and could represent a clonal population. The number of polymorphic bands per strain was also variable, ranging from one to four for *Maloideae* strains to ten for the *Rubus* strain examined. The genetic divergence of *Rubus* strains with respect to *Maloideae* strains has been previously reported in other studies using several molecular techniques, including rep-PCR (20,23,29,32,35). The production of a larger number of polymorphisms by the *Rubus* strain (Table 4) has allowed us to unequivocally differentiate it from the *Maloideae* strains and to provide evidence for its genetic divergence.

The polymorphism revealed by rep-PCR in this work was due to nearly all types of DNA modifications, namely DNA reorganization (bands E4, E5, and R3), base changes in the primer binding sites (band R4) and the existence of DNA that is present in only

some of the strains, either in the chromosome (bands E3, R8) or in a plasmid (bands E2, I4, I6, I7, and P1). Indeed, and in contrast with previous studies (4,29), our results demonstrate for the first time that the possession of pEA29 and other native plasmids can result in the generation of polymorphic bands in rep-PCR assays.

Polymorphic bands E2, I7, and P1 contained DNA specific to a group of 28 strains from Navarre in Northern Spain and hybridized to a cryptic plasmid larger than pEA29, of approximately 35 kb (Fig. 3D). These strains were mostly isolated from two wide areas of the province of Navarre, near the towns of Pamplona and Estella, whereas strains lacking bands E2 and P1 are distributed throughout the province, indicating that the strains containing the 35-kb plasmid might represent a clonal population. Additionally, band I4 (0.6 kb) hybridized to a plasmid present in strain NCPPB311 from Canada, that we have tentatively identified as pEU30 based on its size and the sequence of band I4. According to our dot blot hybridization data and previous results (11), these two native plasmids appear to be present in only a small fraction of the *E. amylovora* population. It is intriguing that the 35-kb plasmid was present only in strains isolated in Navarre, Spain, and pEU30 only in strains isolated from apple or pear in the western United States and Canada, which could indicate that they are good epidemiological markers for tracing spread of the pathogen. A potential limitation is that both plasmids might be transferable because they contain DNA homologous to genes involved in conjugal transfer (Table 5) (11). Although we do not have data from the other strains, strains UPN529 and UPN546, which contain the 35-kb plasmid and were isolated from *Pyracantha* sp. and *Cydonia* sp. plants separated by around 16 km by road, might have a clonal origin because they showed an identical AFLP profile with different primer combinations (41). Interestingly, we detected the existence of sequence variations in the 35-kb plasmids, because only strain UPN526 produced band I7 and a specific amplicon using a primer pair internal to this band, although all the strains containing the 35-kb plasmid showed hybridization to band I7. The availability of the PCR primer pairs designed in this work would undoubtedly be of help to study the distribution of these plasmids in the pathogen population, although it would be necessary to ascertain the relationships among the strains containing them by appropriate techniques, such as AFLP.

Five of eleven polymorphic bands that were cloned in this work contained DNA that was present in the majority of the strains examined, as shown by dot blot and Southern hybridization (Table 5). These DNA regions that show variations and produce the polymorphism could then be the target for the design of a molecular marker. For example, we designed a pair of primers internal to band R3 that produced an amplification product exclusively for strain NCPPB1819 (Fig. 2), but not for the rest of the strains examined, in spite of them containing DNA homologous to band R3. One of the potential applications of this pair of primers is tracing the dispersal of strain NCPPB1819, or of any other strain containing this marker, in the environment. Furthermore, the differential amplification of bands E4 and E5 revealed a source of diversity on the *E. amylovora* population that can be used in epidemiology. The polymorphism was due to the existence of zero to three repetitions of a 125 nt sequence in the intergenic region of the *rpsU-dnaG* operon; a further source of variance is the existence of single nucleotide polymorphisms among the different repeats. These 125 nt repeats are 65% similar to an ERIC element described in the same position in *Salmonella typhimurium* (15), but absent in *E. coli*, and function as a binding site for the ERIC2 primer. However, the ERIC PCR fingerprint shows no apparent alterations with the variations in the number of 125 nt repeats in the *rpsU-dnaG* region, unless they are all eliminated with the concomitant loss of the ERIC2. Therefore, the variability among *E. amylovora* populations could be examined using appropriate primers bordering this intergenic region, and

this information could be used to follow their dispersal, although it would first be necessary to ascertain the stability of this region over time in a given strain.

Six of the cloned polymorphic bands hybridized with less than 15% of the *E. amylovora* strains examined, indicating that the rep-PCR variability is in many cases due to differences in gene content among strains. Four of these bands were due to the differential presence of two native plasmids, as detailed above, whereas two of the polymorphic bands (E3 and R8) corresponded to chromosomal DNA that was likely acquired through horizontal transfer. This is based on the facts that different genera of bacteria contain DNA homologous to these two bands, as shown in a blastx comparison, and that this DNA is present only in strain SL2162 among all the *E. amylovora* strains examined. It would be of interest to explore what other genes are linked to this DNA and if it contributes to virulence, because horizontally acquired DNA usually confer a selective advantage to the recipient bacterium (22,31,37,47).

Our results also indicate that the little genetic variability revealed by some PCR primers is not adequate for constructing genealogies of *E. amylovora*. Two different phenomena may contribute to reduce the phylogenetic value of *E. amylovora* rep-PCR fingerprinting. In the first place, a single molecular event was associated with more than one polymorphism. For example, the disappearance of band E4 was accompanied by the concomitant presence of band E5 in strain LNPV1099. Likewise, the acquisition of a single plasmid resulted in the appearance of two different polymorphic bands, E2 and P1 in 28 strains and a further polymorphic band, I7, in one of the 28 strains. Secondly, sequence and hybridization data indicated that 5 of 11 bands cloned in this work correspond to DNA located in plasmids, which can be lost or acquired by different bacteria in independent unrelated events. However, the PCR fingerprinting assay has shown to be a reliable tool to identify *E. amylovora*, because the fingerprints were highly conserved among a large number of strains from different locations and hosts. Additionally, rep-PCR has provided a number of molecular markers highly specific for individual strains or for small groups of strains. These markers can be used to follow the spread of the pathogen in natural conditions or after artificial inoculations, as well as to study the epidemiology of plasmids in natural populations of the pathogen. Additionally, the approach we followed here can be adapted for the generation of new molecular markers to follow other strains or populations of the pathogen. We have also shown the existence of additional plasmids in *E. amylovora*, whose characteristics, function, and further distribution are worth investigating.

ACKNOWLEDGMENTS

A. Rico and M. E. Führer contributed equally to this work and should be considered as sharing the position of first author. This paper is dedicated to F. García-Arenal. We thank M. M. López, D. Berra, and M. Borruel for providing *E. amylovora* strains, and to G. Sundin, G. Preston, and T. Osinga for critical reading of the manuscript and helpful suggestions. This research was supported with grants AGL2001-1948-CO2-01 and AGL2004-03143, from the Spanish CICYT. AR was funded by a fellowship from the Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA).

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Arnold, D. L., Athey-Pollard, A., Gibbon, M. J., Taylor, J. D., and Vivian, A. 1996. Specific oligonucleotide primers for the identification of *Pseudomonas syringae* pv. *pisi* yield one of two possible DNA fragments by PCR amplification: Evidence for phylogenetic divergence. *Physiol. Mol. Plant Pathol.* 49:233-245.
- Barionovi, D., Giorgi, S., Stoeger, A. R., Ruppitsch, W., and Scortichini, M. 2006. Characterization of *Erwinia amylovora* strains from different host plants using repetitive-sequences PCR analysis, and restriction fragment length polymorphism and short-sequence DNA repeats of plasmid pEA29. *J. Appl. Microbiol.* 100:1084-1094.
- Bereswill, S., Bugert, P., Bruchmüller, I., and Geider, K. 1995. Identification of the fire blight pathogen, *Erwinia amylovora*, by PCR assays with chromosomal DNA. *Appl. Environ. Microbiol.* 61:2636-2642.
- Bonn, W. G., and van der Zwet, T. 2000. Distribution and economic importance of fire blight. Pages 37-55 in: *Fire Blight. The Disease and its Causative Agent, Erwinia amylovora*. J. L. Vanneste, ed. CAB International, Wallingford, UK.
- Brennan, J. M., Doohan, F. M., Egan, D., Scanlan, H., and Hayes, D. 2002. Characterization and differentiation of Irish *Erwinia amylovora* isolates. *J. Phytopathol.* 150:414-422.
- Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* 16:10881-10890.
- Desmarais, E., Lanneluc, I., and Lagnel, J. 1998. Direct amplification of length polymorphisms (DALP), or how to get and characterize new genetic markers in many species. *Nucleic Acids Res.* 1998:1458-1465.
- Donat, V., Biosca, E. G., Peñalver, J., and López, M. M. 2007. Exploring diversity among Spanish strains of *Erwinia amylovora* and possible infection sources. Published Online. *J. Appl. Microbiol. Early Articles*. doi:10.1111/j.1365-2672.2007.03399.x.
- Donat, V., Biosca, E. G., Rico, A., Peñalver, J., Borruel, M., Berra, D., Basterretxea, T., Murillo, J., and López, M. M. 2005. *Erwinia amylovora* strains from outbreaks of fire blight in Spain: Phenotypic characteristics. *Ann. Appl. Biol.* 146:105-114.
- Foster, G. C., McGhee, G. C., Jones, A. L., and Sundin, G. W. 2004. Nucleotide sequences, genetic organization and distribution of pEU30 and pEL60 from *Erwinia amylovora*. *Appl. Environ. Microbiol.* 70:7539-7544.
- Gorris, M. T., Cambra, M., Llop, P., López, M. M., Lecomte, P., Chartier, R., Paulin, J. P., and Bonn, W. G. 1996. A sensitive and specific detection of *Erwinia amylovora* based on the ELISA-DASI enrichment method with monoclonal antibodies. *Acta Hort.* 411:41-46.
- Gürtler, V., and Mayall, B. C. 2001. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int. J. Syst. Evol. Microbiol.* 51:3-16.
- Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A., Nieto, R., Woods, C., Versalovic, J., and Lupski, J. R. 2005. Microbial DNA typing by automated repetitive-sequence-based PCR. *J. Clin. Microbiol.* 43:199-207.
- Hulton, C. S., Higgins, C. F., and Sharp, P. M. 1991. ERIC sequences: A novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Mol. Microbiol.* 5:825-834.
- Ishimaru, C., and Klos, E. J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* 74:1342-1345.
- Jock, S., and Geider, K. 2004. Molecular differentiation of *Erwinia amylovora* strains from North America and two Asian pear pathogens by analyses of PFGE patterns and *hrpN* genes. *Environ. Microbiol.* 6:480-490.
- Jock, S., Jacob, T., Kim, W. S., Hildebrand, M., Vosberg, H. P., and Geider, K. 2003. Instability of short-sequence DNA repeats of pear pathogenic *Erwinia* strains from Japan and *Erwinia amylovora* fruit tree and raspberry strains. *Mol. Genet. Genomics* 268:739-749.
- Jock, S., V. D., López, M. M., Bazzi, C., and Geider, K. 2002. Following spread of fire blight in Western, Central and Southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. *Environ. Microbiol.* 4:106-114.
- Kim, J. H., Beer, S. V., Zumoff, C. H., Laby, R. J., Gustafson, H. L., Aldwinckle, H. S., and Tanii, A. 1996. Characterization of *Erwinia amylovora* strains from different hosts and geographical areas. *Acta Hort.* 411:183-185.
- Kim, W. S., and Geider, K. 1999. Analysis of variable short-sequence DNA repeats on the 29 kb plasmid of *Erwinia amylovora* strains. *Eur. J. Plant Pathol.* 105:703-713.
- Koonin, E. V., Makarova, K. S., and Aravind, L. 2001. Horizontal gene transfer in prokaryotes: Quantification and classification. *Annu. Rev. Microbiol.* 55:709-742.
- Laby, R. J., and Beer, S. V. 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 and DNA of other bacteria. *Mol. Plant-Microbe Interact.* 5:412-419.
- Llop, P., Donat, V., Rodriguez, M., Cabrefiga, J., Ruz, L., Palomo, J. L., Montesinos, E., and Lopez, M. M. 2006. An indigenous virulent strain of *Erwinia amylovora* lacking the ubiquitous plasmid pEA29. *Phytopathology* 96:900-907.

25. López, M. M., Gorris, M. T., Llop, P., Cambra, M., Borruel, M., Plaza, B., Roselló, M., García, P., Palomo, J. L., and Berra, D. 1999. Fire blight in Spain: Situation and monitoring. *Acta Hortic.* 489:187-192.
26. López, M. M., Llop, A., Rico, A., Ortiz, A., Murillo, J., Donat, J., Penalver, I., Llorente, I., Badosa, E., and Montesinos, E. 2002. Chronicle of a disease foretold (that advances slowly): The 2001 Spanish situation. *Acta Hortic.* 590:35-38.
27. Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of pytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* 60:2286-2295.
28. McGhee, G. C., Schnabel, E. L., Maxson-Stein, K., Jones, B. V. S., Lacy, G. H., and Jones, A. L. 2002. Relatedness of chromosomal and plasmid DNAs of *Erwinia pyrifoliae* and *Erwinia amylovora*. *Appl. Environ. Microbiol.* 68:6182-6192.
29. McManus, P. S., and Jones, A. L. 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. *Phytopathology* 85:618-623.
30. McManus, P. S., and Jones, A. L. 1995. Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and *Rubus* spp. *Phytopathology* 85:1547-1553.
31. McManus, P. S., and Jones, A. L. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. *Phytopathology* 84:627-633.
32. Momol, E. A., Momol, M. T., Norelli, J., Beer, S. V., Burr, T. J., and Aldwinckle, H. S. 1999. Relatedness of *Erwinia amylovora* strains based on amplified 16S-23S ribosomal DNA restriction enzyme analysis-ARDREA. *Acta Hortic.* 489:55-59.
33. Momol, M. T., and Aldwinckle, H. S. 2000. Genetic diversity and host range of *Erwinia amylovora*. Pages 55-72 in: *Fire Blight. The Disease and its Causative Agent, Erwinia amylovora*. J. L. Vanneste, ed. CAB International, Wallingford, UK.
34. Momol, M. T., Momol, E. A., Lamboy, W. F., Norelli, J., Aldwinckle, H. S., and Beer, S. V. 1995. Genetic diversity of *Erwinia amylovora* strains as determined by RAPD fragments. *Phytopathology* 85:1158.
35. Momol, M. T., Momol, E. A., Lamboy, W. F., Norelli, J. L., Beer, S. V., and Aldwinckle, H. S. 1997. Characterization of *Erwinia amylovora* strains using random amplified polymorphic DNA fragments (RAPDs). *J. Appl. Microbiol.* 82:389-398.
36. Moulton, P. J., Vivian, A., Hunter, P. J., and Taylor, J. D. 1993. Changes in cultivar-specificity toward pea can result from transfer of plasmid RP4 and other incompatibility group P1 replicons to *Pseudomonas syringae* pv. *pisi*. *J. Gen. Microbiol.* 139:3149-3155.
37. Palmer, E. L., Teviotdale, B. L., and Jones, A. L. 1997. A relative of the broad-host-range plasmid RSF1010 detected in *Erwinia amylovora*. *Appl. Environ. Microbiol.* 63:4604-4607.
38. Paulin, J. P., and Samson, R. 1973. Le feu bactérien en France. II. Caractères des souches d'*Erwinia amylovora* (Burrill) Winslow *et al.* 1920 isolées du foyer Franco-Belge. *Ann. Phytopathol.* 5:389-397.
39. Pooler, M. R., Ritchie, D. F., and Hartung, J. S. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Appl. Environ. Microbiol.* 62:3121-3127.
40. Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., and de Bruijn, F. J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *Int. J. Syst. Evol. Microbiol.* 50:665-677.
41. Rico, A., Ortiz, A., Ritter, E., and Murillo, J. 2004. Genetic characterization of *Erwinia amylovora* isolates by amplified fragment length polymorphism (AFLP). *J. Appl. Microbiol.* 96:302-310.
42. Rodriguez, R. J., and Yoder, O. C. 1991. A family of conserved repetitive DNA elements from the fungal plant pathogen *Glomerella cingulata* (*Colletotrichum lidemuthianum*). *Exp. Mycol.* 15:232-242.
43. Rozen, S., and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365-386 in: *Bioinformatic Methods and Protocols: Methods in Molecular Biology*. S. Krawetz, and S. Misene, eds. Humana Press, Totowa, NJ.
44. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
45. Srimanote, P., Paton, A. W., and Paton, J. C. 2002. Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative shiga-toxicogenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun.* 70:3094-3100.
46. Starr, M. P., Cardona, C., and Folsom, D. 1951. Bacterial fire blight of raspberry. *Phytopathology* 41:915-919.
47. Sundin, G. W., and Bender, C. L. 1996. Molecular analysis of closely related copper- and streptomycin-resistance plasmids in *Pseudomonas syringae* pv. *syringae*. *Plasmid* 35:98-107.
48. Sundin, G. W., and Murillo, J. 1999. Functional analysis of the *Pseudomonas syringae* *ruAB* determinant in tolerance to ultraviolet B (290-320 nm) radiation and distribution of *ruAB* among *P. syringae* pathovars. *Environ. Microbiol.* 1:75-88.
49. Taylor, J. D., Teverson, D. M., Allen, D. J., and Pastor-Corrales, M. A. 1996. Identification and origin of races of *Pseudomonas syringae* pv. *phaseolicola* from Africa and other bean growing areas. *Plant Pathol.* 45:469-478.
50. Trébaol, G., Manceau, C., Tirilly, Y., and Boury, S. 2001. Assessment of the genetic diversity among strains of *Xanthomonas cynarae* by randomly amplified polymorphic DNA analysis and development of specific characterized amplified regions for the rapid identification of *X. cynarae*. *Appl. Environ. Microbiol.* 67:3379-3384.
51. Weising, K., Weigand, F., Driesel, A. J., Kahl, A. J., Zischer, H., and Epplen, J. T. 1989. Polymorphic simple GATA/GACA repeats in plant genomes. *Nucleic Acids Res.* 17:10128.
52. Zhang, Y., and Geider, K. 1997. Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* 63:4421-4426.