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Short communication

Identification by PCR analysis on plasmid pEA29 of isolates of *Erwinia amylovora* responsible of an outbreak in Central Europe

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Abstract

A collection of 127 strains of *Erwinia amylovora*, the causative agent of fire blight, was tested by PCR amplification of a fragment of the plasmid pEA29. A variability in the length of the DNA fragment obtained was observed after digestion by *Msp*I and *Sau*3A restriction enzymes. Strains were distributed into three groups according to the length of the DNA product. Most of the strains analysed were placed into two groups. Thirteen strains were clustered into a third group which was linked with the geographical origin of strains: they were all isolates from recently reported outbreaks of fire blight in Austria and in southern Bavaria in Germany. The variation in the length of the amplified fragment is probably due to an insertion into this fragment.

Introduction

Erwinia amylovora is a Gram-negative bacterium which causes the fire blight disease on pome fruit trees and other rosaceous hosts. This necrotic disease is sporadic but it can be very destructive for some very susceptible genotypes of *Maloideae*. Epidemic spread of fire blight results in a continual expansion of this disease (Vanneste, 1995). It has been reported throughout the main apple and pear producing regions in North and Central America, in Europe, in New Zealand, in Egypt and in Western Asia. The last countries in Europe, where fire blight was reported for the first time were Austria in 1993 (Keck, 1994) and Hungary in 1996 (Anonymous, 1996). In Austria, a restricted outbreak has been found in the western part (Vorarlberg region), close to the German border and near the Lake Constance.

Prophylactic measures are needed to prevent or limit the spread of this quarantine pathogen into fire blight-free areas. Many conventional methods of detection and identification are used for routine diagnosis. However, health inspection services are always interested in more rapid, more sensitive and more specific procedures readily available such as molecular-based methods which have been developed for its effective detection (Falkenstein et al., 1988; Hale and Clark, 1990; Bereswill et al., 1992; McManus and Jones, 1995).

Bereswill et al. (1992, 1993) were the first to propose a procedure of detection of *Erwinia amylovora* by a polymerase chain reaction analysis (PCR). This method was based on the amplification of a fragment of the plasmid pEA29 which is common to *E. amylovora* strains (Falkenstein et al., 1989; Laurent et al., 1989). This fragment was supposed to be the 0.9 kb *Pst*I fragment G according to the physical map of the plasmid pEA29 (Bereswill et al., 1993). But recently, McManus and Jones (1995) indicated that this PCR product is more likely to be a portion of the 1.1 kb *Pst*I fragment F than the 0.9 kb *Pst*I fragment G. Whether fragment F or G is amplified does not affect the usefulness of the identification method (McManus and Jones, 1995).

Until now, *Erwinia amylovora* has been considered a homogeneous species. No major characteristics have been found that can be used to distinguish strains

from different geographic origin and no restriction fragment length polymorphism has been recorded so far (Vanneste, 1995).

In the present study, we describe the identification of a collection of strains of *Erwinia amylovora* by polymerase chain reaction (PCR) according to the procedure proposed by Bereswill et al. (1992). Then, we show a variation between strains in the length of the amplified fragment which is confirmed after DNA digestion. Finally a relationship between a geographical origin and a typical group of strains is pointed out.

Materials and methods

Bacterial strains and preparation. A total of 127 strains of *E. amylovora* was studied. They are listed and described in Table 1. Most were isolated from European countries. Each strain was grown by plating on King's medium B (King et al., 1954) and incubated for 24–36 h at 26 °C.

DNA amplification. Bacterial suspensions were prepared in sterile distilled water. Concentration was approximately adjusted to 3×10^8 colony forming units per ml by comparison of turbidity of suspensions against a Mc Farland's reference scale tube (Klement, 1990). 10 μl samples were placed into 0.65 ml thin-walled PCR tubes (Sorenson^{TM}, BioScience, Inc., Utah). DNA was denatured in a programmable thermal controller (PTC-150, MJ Research^{TM}, Inc., Watertown, MA) at 95 °C for 10 min followed by 5 to 10 min at 4 °C. A PCR-reaction mixture (40 μl) was added to each sample. The final PCR-reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 6 mM $MgCl_2$, 0.01% Tween 20, 350 μM of each dATP, dCTP, dGTP, dTTP (Eurogentec, Seraing, Belgium), 2.5 units of *Euro*Taq DNA polymerase (Eurogentec), and 33 pmoles of each primer A and B. Primers were synthesized by Eurogentec according to the sequences published by Bereswill et al. (1992): 5′-CGGTTTTTAACGCTGGG (primer A) and 5′-GGGCAAATACTCGGATT (primer B) and stored in aliquots at −20 °C. The mixture was overlaid with light mineral oil. Amplification was achieved in the programmable thermal controller, after a predenaturation step at 92 °C for 1 min, by 35 incubation cycles of denaturation at 92 °C for 1 min, primer annealing at 52 °C for 1 min and polymerization at 72 °C for 1 min. Final DNA extension was performed at 72 °C for 2 min before unlimited incubation at 10 °C. Sterile water was used as negative control. Aliquots of PCR products (10 μl) were analysed by horizontal electrophoresis (30 min at 100 V) through agarose gel: 1% (w/v) Seakem^{TM} and 2% (w/v) NuSieve^{TM} (FMC BioProducts, Rockland, ME) in Tris-acetate buffer (Maniatis et al., 1982). 0.35 μg of a 100-bp DNA ladder marker (Gibco BRL, Life Technologies, Inc.) was used as a size marker. Then amplification products were stained with ethidium bromide and photographed after visualisation with UV light (364 nm).

Restriction fragment analysis. Aliquots of forty microliters of PCR products were supplemented with 4 μl of 3M sodium acetate buffer and 100 μl of ethanol at −20 °C. DNA products were precipitated at −20 °C for 30 min, centrifugated at 13000 rpm for 5 min. Pellets were then resuspended in 35 μl of sterile water. 8 μl aliquots of DNA were digested with the following restriction endonucleases; *Alu*I, *Hae*III, *Hha*I, *Msp*I, *Pst*I, *Rsa*I and *Sau*3A for 1 to 2 h with 5 U of enzyme in a 12 μl volume at 37 °C. The whole sample was electrophoresed in a 2.5 to 3.5% (w/v) agarose gel (Metaphor ^{TM}, FMC BioProducts, Rockland, ME) in Tris-borate buffer (Maniatis et al., 1982), and analysed with the same size marker as mentioned above. PCR products of strains CFBP 1430, 295/93 or 296/93, and Ea 115 were used as experiment controls in all electrophoreses.

Results and discussion

PCR analysis. All strains were tested at least twice. All excepted one strain, PMV 6014, gave a single DNA fragment after PCR. This strain is a mutant of CFBP 1430 cured of the plasmid pEA29 (Laurent et al., 1989). It did not give any amplification product, as expected. Three other mutants of the strain CFBP 1430 were used (Table 1): they are similar to the parental strain excepted that they contained a Mu*d*IIPR13 insertion in *hrp* genes. Similar DNA fragments were always obtained for these strains, which beared the same plasmid, indicating that the amplification was reliable for similar DNA target whatever the strain used.

In the electrophoresis conditions, as shown on Figure 1, the size of amplified fragments after PCR amplification was always larger than 900 bp reported by Bereswill et al. (1993). This result corroborates other recent results (as McManus and Jones, 1995).

Table 1. *Erwinia amylovora* strains and relevant characteristics

Strain designations*	Country and year	Host plant	Source/reference	Group
295/93	Austria, 1993	*Cotoneaster salicifolius*	M. Keck	3
296/93	" , 1993	*Cotoneaster salicifolius*	"	3
674/94	" , 1994	*Pyrus communis*	"	3
169/95	" , 1995	*Malus x domestica*	"	3
172/95	" , 1995	*Pyrus communis*	"	3
176/95	" , 1995	*Pyrus communis*	"	3
178/95	" , 1995	*Pyrus communis*	"	3
179/95	" , 1995	*Pyrus communis*	"	3
180/95	" , 1995	*Malus x domestica*	"	3
182/95	" , 1995	*Malus x domestica*	"	3
LBP 93/140	Germany, Motzach, 1993	*Malus* sp.	G. Poschenrieder	3
LBP 93/141	"	*Pyrus* sp.	"	3
LBP 93/400	" , Dittelbrunn, 1993	*Cotoneaster*	"	2
LBP 94/378	" , Neu-Ulm, 1994	*Pyrus* sp.	"	2
DL1	" , Lindau, 1994	*Malus x domestica*		3
Ea 7/74	" , Dossenheim	*Cotoneaster bullatus*	W. Zeller	1
Ea 91/R2	" , Potsdam, 1985	*Pyrus* sp.	K. Richter	1
Ea 115	" , Eisleben, 1989	*Malus x domestica*	"	2
Ea 243	" , Francfort, 1994	*Malus* sp.	"	1
CFBP 1376 (XIII)	Belgium 1972	*Cotoneaster* sp.	R. Veldeman	2
CFBP 3014	" , 1980	*P. com.* cv. Durondeau	R. Vantomne	1
FG-2	Bulgaria	*P. com.* cv. Passe-Crassane	R. Penev	1
T21-3	"	*P. com.* cv. Passe-Crassane	"	1
CFBP 1261	Canada	*Malus x domestica*	R. Layne	1
CFBP 3049 (CUCPB 070)	" , Ontario	*M. x dom.* cv. Jonathan	W.G. Bonn *via*	1
CFBP 3050 (CUCPB 071)	" , Ontario, 1980	*M. x dom.* cv. R.I. Green	J.L. Norelli	2
CFBP 1398 (220)	Denmark	*Crataegus* sp.	J. Bech-Andersen	1
CFBP 1399 (126)	"	*Malus sylvestris*	"	1
PMV 6014	France		Laurent et al., 1989	–
PMV 6023	"		Barny et al., 1990	1
PMV 6046	"		Barny et al., 1990	1
PMV 6086	"		Tharaud et al., 1994	1
CFBP 1430	" , Nord, 1972	*Crataegus* sp.	R. Samson	1
CFBP 2301	" , 1981	*Pyracantha* sp.	J.F. Chauveau	1
CFBP 3041 (NCPPB 1665)	"	*Malus florentina*		1
2543P	" , Angers, 1992	*M. x dom.* 'Lancep'	P. Lecomte	1
2544P	" , Limousin, 1992	*Malus* sp.	"	2
2545P	" , Trémentines, 1992	*M. x dom.* 'M9'	"	1
2546P	" , Lafitte/Lot, 1992	*M. x dom.* 'M9'	"	1
2547P	" , Angers, 1992	*M. x dom.* 'M9'	"	1
2548P	" , Lafitte/Lot, 1992	*M. x dom.* 'M9'	"	2
2549P	" , Angers, 1992	*Malus* sp.	"	1
2550P (CFBP 3472)	" , Angers, 1992	*M. x dom.* 'M26'	"	1
2552P	" , Angers, 1992	*M. x dom.* cv. Idared	"	1
2581P	" , Moissac, 1992	*Malus* sp.	"	1

Table 1. Continued

Strain designations*	Country and year	Host plant	Source/reference	Group
2582P	" , Moissac, 1992	*Malus* sp.	"	1
2590P	" , Objat, 1993	*P. com.* cv. Conférence	"	1
2592P	" , Moissac, 1993	*Malus* sp.	"	1
2592 (2)P	" , Moissac, 1993	*Malus* sp.	"	2
3-7 (LNPV 93-1613)	" , Niort, 1993	*Pyracantha* sp.	C. Audusseau	1
3-8 (LNPV 93-1594)	" , Damp. en B., 1993	*Cotoneaster dammeri*	"	1
3-9 (LNPV 93-1601)	" , Lyon, 1993	*Pyrus communis*	"	1
3-10 (LNPV 93-1626)	" , Lyon, 1993	*Pyrus communis*	"	1
3-15 (LNPV 93-1778)	" , Montreuil, 1993	*Cotoneaster salicifolius*	"	1
6-11 (LNPV 92-1392b)	" , Plesse, 1992	*Malus pumila*	"	1
6-15 (LNPV 92-755b)	" , 1992	*Pyrus pyrifolia*	"	1
6-17 (LNPV 92-810c)	" , Allonnes, 1992	*Pyrus* sp.	"	1
6-20 (LNPV 92-901)	" , Fleury les A., 1992	*Cotoneaster salicifolius*	"	1
6-45 (LNPV 92-1574)	" , Lyon, 1992	*Cydonia oblonga*	"	2
6-67 (LNPV 91-962)	" , Coulonge, 1991	*Crataegus monogyna*	"	1
7-15 (LNPV 91-1145)	" , Angers, 1991	*Pyrus communis*	"	1
7-24 (LNPV 91-1190)	" , La Rochelle, 1991	*Pyracantha* sp.	"	1
7-32 (LNPV 91-1515A)	" , Ville d'Anse, 1991	*Cotoneaster salicifolius*	"	1
7-37 (LNPV 91-839A)	" , 1991	*Malus* sp.	"	1
8-70 (LNPV 94-1087)	" , Lyon, 1994	*Pyrus* sp.	"	1
8-75 (LNPV 94-1278)	" , Reignac, 1994	*Malus pumila*	"	1
9-7 (LNPV 94-1416)	" , Toulouse, 1994	*Pyrus communis*	"	1
9-10 (LNPV 94-1406B)	" , Montemboeuf, 1994	*Pyrus salicifolia*	"	1
9-25 (LNPV 94-1643A)	" , Lyon, 1994	*Cotoneaster dammeri*	"	1
9-43 (LNPV 94-1870)	" , Strasbourg, 1994	*Cotoneaster horizontalis*	"	2
9-44 (LNPV 94-1852)	" , Niort, 1994	*Mespilus germanica*	"	1
9-45 (LNPV 94-1831)	" , Rennes, 1994	*Pyracantha* sp.	"	1
9-61 (LNPV 94-2028)	" , Strasbourg, 1994	*Sorbus* sp.	"	2
9-62 (LNPV 94-2029)	" , Strasbourg, 1994	*Crataegus* sp.	"	2
9-70 (LNPV 94-2159)	" , Doué la F., 1994	*Cotoneaster salicifolius*	"	1
9-75 (LNPV 94-2143)	" , Rennes, 1994	*Cotoneaster watereri*	"	1
11-49 (LNPV 94-3644)	" , Colmar, 1994	*Cotoneaster* sp.	"	1
11-51 (LNPV 94-3556A)	" , Prigonrieux, 1994	*Pyrus communis*	"	1
11-52 (LNPV 94-3557A)	" , 1994	*Malus pumila*	"	1
BPIC-850	Greece, 1985	*P. com.* cv. Passe-Crassane	N. Alivizatos	1
BPIC-883	" , 1988	*P. com.* cv. Passe-Crassane	"	1
BPIC-912	" , 1991	*P. com.* cv. Abbé Fétel	"	1
BPIC-913	" , 1991	*P. com.* cv. De Cayan	"	1
BPIC-915	" , 1991	*P. com.* cv. Kondoula	"	1
BPIC-919	" , 1991	*Pyrus amygdaliformis*	"	2
BPIC-926	" , 1992	*P. com.* cv. Général Leclerc	"	1
BPIC-931	" , 1993	*M. x dom.* cv. G. Smith	"	1
BPIC-950	" , 1995	*Crataegus* sp.	"	1
A 450 (CFBP 3465)	" , Volos, 1994	*Pyrus amygdaliformis*	J. Tsiantos	2
A 458 (CFBP 3466)	" , Mt Pilion, 1994	*Malus x domestica*	"	2
A 461 (CFBP 3467)	" , Mt Pilion, 1994	*Malus x domestica*	"	2
2610P	" , 1994		"	2
2611P	" , 1994		"	2
2612P	" , 1994		"	2

Table 1. Continued

Strain designations*	Country and year	Host plant	Source/reference	Group
CFBP 2584	Ireland, 1986	*Cotoneaster* sp.	J.P. Paulin	1
CFBP 2585	" , 1986	*Sorbus* sp.	"	1
CFBP 3096	Israël	*Cydonia ablonga*		2
CFBP 3098	" , 1987	*M. x dom.* cv. Ein-Shemer	D. Zutra	1
CFBP 1234 (NCPPB 1951)	The Netherlands, 1966	*Pyrus communis*	H.P. Maas Geesteranus	2
CFBP 3017	" , 1973	*Crataegus* sp.		2
CFBP 3019	"	*Prunus laurocerasus*		1
CFBP 3020	" , 1981	*P. com.* cv. Clapp's Favour		2
CFBP 3021	" , 1981	*Pyrus communis*		2
3-36 (LNPV PD 437)	" , 1981	*P. com.* cv. Clapp's Favour	J.D. Janse	2
CFBP 1225 (NCPPB 2083)	New Zealand, 1963	*Malus sylvestris*	W.J. Kemp	1
CFBP 1226 (NCPPB 2086)	" , 1963	*Crataegus* sp.	"	1
CFBP 1217 (S2)	Poland	*Crataegus* sp.	A. Burkowicz	1
CFBP 1218 (S3C)	"	*Crataegus* sp.	"	1
CFBP 1222 (S10)	"	*Malus* sp.	"	1
CFBP 1196 (NCPPB 595)	United Kingdom, 1958	*Pyrus communis*	J.E. Crosse	1
CFBP 1197 (NCPPB 742)	" , 1959	*Crataegus* sp.	E. Billing	2
CFBP 1232 (NCPPB 683)	" , 1959	*Pyrus communis*	J.E. Crosse	1
CFBP 3042 (NCPPB 1666)	"	*Pyrus calleryana*		2
CFBP 1228 (FB1)	United States, 1967	*Cotoneaster* sp.	M.N. Schroth	2
CFBP 1229 (FB9)	" , 1966	*Malus sylvestris*	"	2
CFBP 2150	"	*Rubus*	J. Klopmeyer	1
CFBP 3051 (CUCPB 273)	" , NY, 1980	*M. x dom.* cv. R.I. Green	S.V. Beer *via* J.L. Norelli	1
CFBP 2582	Sweden	*Pyrus* sp.		1
353-90	Switzerland, 1990	*Cotoneaster salicifolius*	W. Vogelsanger	1
41-91	" , 1991	*Cotoneaster dammeri*	"	1
511-94	" , 1994	*Malus* sp.	"	2
517-94	" , 1994		"	2
C5	Syria	*Cydonia* sp.		1
CFBP 3468 (1)	Turkey, Antalya	*Pyrus* sp.	H. Saygili	2
CFBP 3469 (10)	" , Yalova	*Pyracantha* sp.	"	2
CFBP 3470 (TPBB-3204)	" , Ankara	*Malus* sp.	"	2
CFBP 3471 (TPBB-3404)	" , Ankara	*Cydonia* sp.	"	2

* Lab designation or national collection designation. Abbreviations:
BPIC: Benaki Phytopathological Institute Collection; CFBP: Collection Française de Bactéries Phytopathogènes; CUCPB: Cornell University Collection of Phytopathogenic Bacteria; LBP: Bayerische Landesanstalt für Bodenkultur und Planzenbau; LNPV: Laboratoire National de la Protection des Végétaux – Angers; NCPPB: National Collection of Plant Pathogenic Bacteria; PMV: Laboratoire de Pathologie Moléculaire et Végétale – Paris.

In addition, a variability in the size of bands was observed: for example, strains 295/93, 296/93, 674/94, DL1 and A 450 (respectively lanes E to H and lane J, Figure 1) exhibited a fragment larger than those of other strains. After this observation, a restriction analysis was undertaken to further investigate the differences between strains.

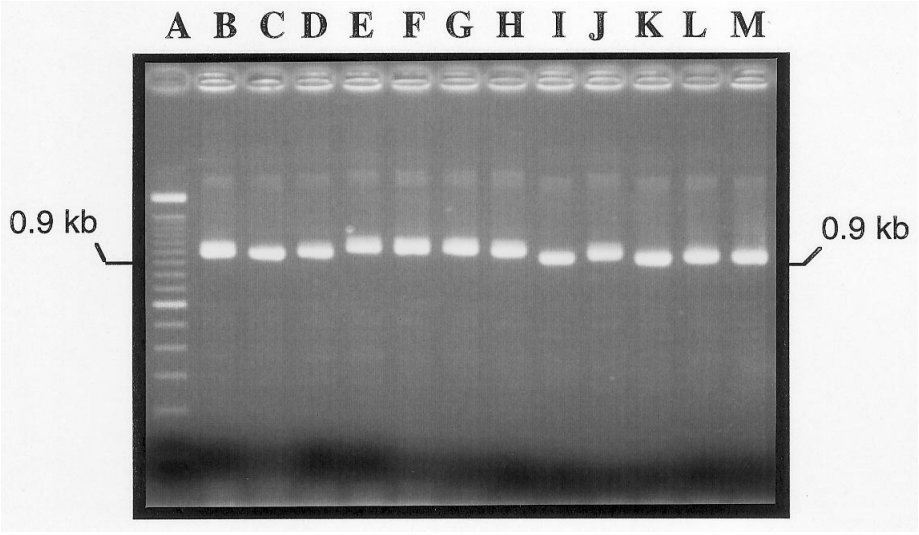

Figure 1. PCR amplification products with cells of various *E. amylovora* strains. Cells from 24–36 h pure culture (3×10^8 bacteria per ml) were applied to the PCR assay. Lane A, 100-bp ladder; lane B, strain C5; lane C, strain EA 91; lane D, strain EA 115; lane E, strain 295/93; lane F, strain 296/93; lane G, strain 674/94; lane H, strain DL 1; lane I, strain CFBP 1430; lane J, strain A 450; lane K, strain CFBP 3098; lane L, strain CFBP 1399; lane M, strain CFBP 2582. The position of 0.9 kb band is indicated.

RFLP analysis. Seven restriction enzymes were tested. They all recognize tetranucleotide target sequences excepted *Pst*I. The DNA from the following selection of strains was digested with the whole set of enzymes: C5, Ea 91/R2, Ea 115, 295/93, 296/93, 674/94, DL1, A450, A458, A461, T21-3, CFBP 1222, CFBP 1234, CFBP 1399, CFBP 1430, CFBP 2150, CFBP 2582, CFBP 2584, CFBP 3017, CFBP 3042, CFBP 3049, CFBP 3051 and CFBP 3098. *Alu*I, *Hae*III, *Hha*I, *Pst*I, and *Rsa*I did not produce any cleavage fragments. Conversely, *Msp*I and *Sau*3A cleaved the amplified fragment and were used to digest the PCR products of all other *E. amylovora* strains tested (Table 1).

After digestion with *Msp*I, two DNA fragments had the same size whatever the strain (330 bp, 195 bp). There were probably two small fragments whose size was interpolated to 50 bp. The size of the larger fragment was either 370 bp, about 400 bp or 450 bp, according to the strains (Figure 2).

Similarly, after digestion with *Sau*3A, five DNA fragments had the same size (two fragments very close in size about 180 bp, 125 bp, 115 bp, 110 bp). There were also probably two small fragments whose size was interpolated to 20 bp. Another fragment, also the larger one, showed a size which was either 250 bp, between 280 bp and 290 bp, or 340 bp, according to the strains (Figure 2).

When digested with these latter enzymes, the restriction sites of the amplified fragment were similar for all *E. amylovora* strains tested. But one restriction fragment, the larger one in both cases, is still further larger for certain strains, indicating that an insertion of DNA equal to 30 bp to 90 bp occurred in this region.

Three groups were distinguished among all tested strains, according to the length of the largest restriction fragment (Table 1). Group 1 gathered strains exhibiting the shortest fragment. The French standard wild type strain, CFBP 1430, is representative of this group with a distinctive fragment of about 370 bp and 250 bp with *Msp*I and *Sau*3A respectively (Figure 2, lane E). In group 2, strains exhibited intermediate and variable size DNA fragment of about 400 bp and about 290 bp when digested with *Msp*I and *Sau*3A respectively (e.g. strains EA 115, CFBP 1376 and CFBP 3042 as shown on Figure 2, lanes B, C and D). Subgroups could be distinguished within this group 2 as some strains showed fragments slightly different than others. For example, strain EA 115 provided a fragment slightly larger than CFBP 1376 (Figure 2, lanes B and C) and, A450, from Greece, gave a fragment intermediate in size between those of group 2 and those of group 3 (result not shown). This suggests that a noticeable variability might be found between isolates. Group 3 clustered strains which exhibited the largest fragment.

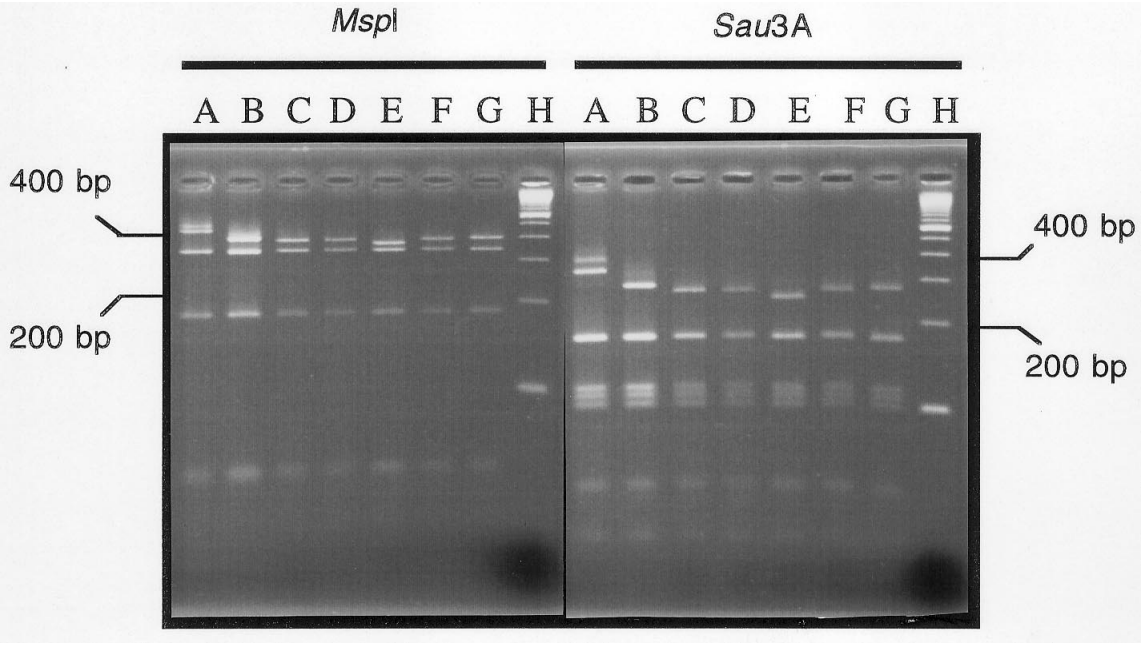

Figure 2. Cleavage patterns of the specific amplification products from plasmid pEA29 after digestion with *Msp*I and *Sau*3A enzymes respectively. The strain are distributed in three groups according to the size of the largest restriction fragment. Lane A, strain 296/93 (group 3); lane B, strain Ea 115 (group 2); lane C, strain CFBP 1376 (group 2); lane D, strain CFBP 3042 (group 2); lane E, strain CFBP 1430 (group 1); lane F, strain CFBP 1234 (group 2); lane G, strain CFBP 3096 (group 2); lane H, 100-bp ladder. The positions of 200 bp and 400 bp bands are indicated for each digestion assay.

A representative strain of this group is strain 296/93 (Figure 2, lane A) with a distinctive fragment of about 450 bp and about 340 bp when digested with *Msp*I and *Sau*3A respectively.

Out of 126 strains, 78 were placed into group 1 (62%) and 35 in group 2 (28%). Thirteen only were ranged in group 3 (10%). No relationship between a group and a host plant species was observed. Strains belonging to groups 1 and 2 were worldwide distributed. Conversely, strains belonging to the group 3 were isolated in a restricted area, recently infected, near the Lake Constance. Three of them (93/140, 93/141 and DL1) were isolated in southern Bavaria in 1993 and 1994. The remaining strains were isolated in Austria, just close to the German border, in 1993, 1994 and 1995. These strains were accurately identified by physiological, serological and pathogenicity tests and no biological difference was found in comparison with other European strains (Keck et al., 1996).

Therefore, these results could suggest that infections recorded in South Bavaria and West Austria resulted from the same outbreak of fire blight. The polymorphism in the length of amplified DNA fragment allowed to mark the bacterial population responsible for this outbreak. All bacterial strains belonging to this group 3 were isolated very recently, that could indicate that the mutation, which is probably due to an insertion of a nucleotide sequence, occurred recently. Consequently it was not possible to distinguish the origin of bacterial population responsible of this given outbreak. However, the identified polymorphism could be used now as an epidemiological marker to trace the progress of the disease in this area of Europe.

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