

SHORT COMMUNICATION

MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAINS FROM DIFFERENT HOST PLANTS USING FLUORESCENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISMG. Cirvilleri¹, G. Scuderi¹, A. Bonaccorsi¹, M. Scortichini²¹ Dipartimento di Scienze e Tecnologie Fitosanitarie, Università degli Studi, Via S. Sofia 100, Catania, Italy² C.R.A., Istituto Sperimentale per la Frutticoltura, Via di Fioranello 52, 00040 Ciampino aeroporto, Roma, Italy

SUMMARY

The ability of fluorescent Amplified Fragment Length Polymorphism (fAFLP) technique for typing *Pseudomonas syringae* pv. *syringae* strains isolated from different host plants was investigated. Fragments of up to 700 bp were separated on a CEQ 8000 automated DNA sequencer and electropherograms were generated with CEQ 8000 analysis software.

Different patterns were generated from 57 *P. s.* pv. *syringae* strains examined and seven fAFLP clusters partially corresponding to the host of origin were identified. The analysis of the electropherograms allowed the identification of host-specific discriminative peaks. One peak was present in 93% of the analyzed strains. fAFLP analysis showed a high genetic heterogeneity in the *P. s.* pv. *syringae* strains examined, including strains isolated from the same host.

Key words: fAFLP, molecular typing, *Pseudomonas syringae* pv. *syringae*.

Various molecular techniques have been used to characterize *Pseudomonas syringae* pv. *syringae* strains. They include Pulsed-Field Gel Electrophoresis (PFGE) (Grothues and Rudolf, 1991), Restriction Fragment Length Polymorphism (RFLP) (Scholz *et al.*, 1994), Random Amplified Polymorphic DNA (RAPD) (Clerc *et al.*, 1998), repetitive-sequence PCR (rep-PCR) (Little *et al.*, 1998; Scortichini *et al.*, 2003), Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA) (Scortichini *et al.*, 2001) and Amplified Fragment Length Polymorphism (AFLP) (Clerc *et al.*, 1998; Manceau and Brin, 2003). Presently, AFLP analysis can be considered one of the most discriminating genomic methods to distinguish among bacterial strains (Vos *et al.*, 1995).

In order to reduce the complexity of the original AFLP technique, the AFLP protocol was recently modified by the introduction of fluorescent dye-labeled

primers and use of an automated DNA sequencer for data capture. So far, this improved method (fAFLP) has successfully been used for the identification and/or typing of bacterial species (Manceau and Brin, 2003; Matarante *et al.*, 2004; Roumagnac *et al.*, 2004; Boudon *et al.*, 2005).

To determine whether fAFLP analysis is appropriate for the characterization of *P. s.* pv. *syringae* strains isolated from different host plants and previously characterized for pathogenic and antagonistic activity and typed using repetitive-sequence PCR (Cirvilleri *et al.*, 2005), we analysed 57 strains of this pathogen (Table 1).

Each strain was grown overnight in 5 ml of nutrient broth (NB) (Oxoid, Basingstoke, UK) at 27°C in a rotating incubator at 170 rpm. Cells were collected by centrifugation at 2500 g, and washed twice with sterile distilled water. Genomic DNAs were extracted and purified using the DNA Purification Kit (Puregene, Gentra, Minneapolis, MN, USA) following the manufacturers' instructions. The extracted DNA was quantified by electrophoresis on 1% agarose gel in the presence of an appropriate marker (High DNA mass ladder, Invitrogen-Life Technologies, Paisley, UK).

DNA template preparation using restriction enzymes *EcoRI* and *MseI* (Invitrogen-Life Technologies, Paisley, UK) and adaptors ligation were carried out as reported by Vos *et al.* (1995). Briefly, bacterial genomic DNA (250 ng) from each strain was digested with 10 U of *EcoRI* and 10 U of *MseI* in 10X reaction buffer for 4 h at 37°C in a final restriction volume of 25 µl. Restriction enzymes were heat-inactivated at 75°C for 15 min. In the same reaction mixture, 5 µl of *EcoRI* and *MseI* adaptors (*EcoRI* forward: 5'-CTCGTAGACTGCGTACC-3', *EcoRI* reverse: 5'-AATTGGTACGCAGTC-TAC-3', *MseI* forward: 5'-GACGATGAGTCCTGAG-3', *MseI* reverse: 5'-TACTCAGGACTCAT-3') were added at final concentrations of 2 and 20 µM, respectively, and ligated for 2 h at 20°C using 2 U of T4 DNA ligase in a 5X ligase buffer.

The final ligation mixture volume was 50 µl. The PCR mixtures (50 µl) consisted of 5 µl of ligated DNA (dilution 1:20 with distilled water), 1 µl of 5 µM *MseI*-primer (5'-GATGAGTCCTGAGTAAC-3'), 1 µl of 5 µM *EcoRI*-primer (5'-GACTGCGTACCAATTCA3') la-

Table 1. Strains of *Pseudomonas syringae* pv. *syringae* used in this study, country of origin and main characteristics.

Strains ^a	Host	Country of origin	Year	Antagonistic group ^b	Patogenicity to lemon ^c
PVCT 10.2	<i>Citrus sinensis</i>	Italy	1990	A	8 ^{b-d}
PVCT 41 ₂	"	"	1990	A	8 ^{b-d}
PVCT 130 ₁	"	"	1990	A	10 ^{cd}
PVCT 147 ₁	"	"	1990	A	8 ^{b-d}
PVCT 281 ₁	"	"	1990	A	5 ^b
PVCT 285 ₁	"	"	1990	A	10 ^{cd}
PVCT 291 ₁	"	"	1990	A	9 ^{b-d}
PVCT 293 ₁	"	"	1990	A	5,0 ^b
PVCT 310	"	"	1990	A	10 ^{cd}
PVCT 334	"	"	2000	A	8 ^{b-d}
PVCT 335 ₂	"	"	2000	A	8 ^{b-d}
PVCT 337 ₁	"	"	2000	A	10 ^{cd}
PVCT 339 ₁	"	"	2000	A	7,5 ^{b-d}
PVCT 48SR1	"	"	1990	A	10 ^{cd}
PVCT 48SR2	"	"	1990	A	10 ^{cd}
ISF 242	<i>C. lemon</i>	"	1996	A	10 ^{cd}
ISF 243	<i>C. reticulata</i>	"	1996	A	12 ^d
PVCT 23P	<i>Pyrus communis</i>	"	1998	A	8 ^{b-d}
PVCT 26P	"	"	1998	A	10 ^{cd}
PVCT 46P	"	"	1998	A	10 ^{cd}
PVCT 76P	"	"	1998	A	10 ^{cd}
ISF 280	"	"	1996	A	9 ^{b-d}
ISF 281	"	"	1996	A	5 ^b
ISF 288	"	"	1996	A	6,5 ^{bc}
PVCT 1.1S	<i>Strelitzia reginae</i>	"	2000	A	4 ^b
PVCT 1.3S	"	"	2000	A	8,5 ^{b-d}
PVCT 1.4S	"	"	2000	A	5,5 ^{bc}
ISF 107=NCPB 1093	<i>Prunus armeniaca</i>	New Zealand		A	0,0 ^a
ISF 231	"	Italy	1996	A	0,0 ^a
ISF 015=NCPB3869	<i>Laurus nobilis</i>	"	1992	A	12 ^d
ISF 282	<i>Castanea sativa</i>	"	1996	A	8 ^{b-d}
AID 33	<i>Fragaria x ananassa</i>	"		B	0,0 ^a
AID 48	"	"	1988	A	12 ^d
AID 76	"	"	1988	B	0,0 ^a
HRI 1480A	<i>Pisum sativum</i>	UK		A	7,0 ^{bc}
ISF 284 =PSS61	<i>Triticum aestivum</i>	USA (D.C. Gross)		B	0,0 ^a
ISF 294 =W451	"	"		B	0,0 ^a
ISF 295 =SD202	"	"		B	0,0 ^a
ISF 292	"	USA (J.E.De Vay)		A	0,0 ^a
ISF 300	"	Italy		A	7,0 ^{bc}
ISF 304	"	"	1996	A	0,0 ^a
ISF 309	"	"	1996	A	8 ^{b-d}
ISF 355	<i>Hordeum vulgare</i>	Italy	1996	A	5,0 ^b
ISF 356	"	"	1996	A	5,0 ^b
ISF 359 =475A	"	USA (J.E.De Vay)		A	12 ^d
ISF 357	"	Italy	1996	B	0,0 ^a
ISF 293 =B359	<i>Setaria italica</i>	Australia (J.E.De Vay)		A	8 ^{b-d}
PVCT 7 NC	"	Italy	2005	A	0,0 ^a
PVCT 44 NC	"	"	2005	A	0,0 ^a
PVCT 38 NC	"	"	2005	B	0,0 ^a
ISF 291 =SY12	<i>Syringa vulgaris</i>	Japan (D.C. Gross)		B	0,0 ^a
PVCT B728a	<i>Phaseolus vulgaris</i>	USA (S.E. Lindow)	1986	B	4,0 ^b
PVCT 4	<i>Cynara scolymus</i>	Italy	1992	B	0,0 ^a
PVCT 40	"	"	1992	B	0,0 ^a
PVCT 106	"	"	1992	B	0,0 ^a
PVCT 120	"	"	1992	B	0,0 ^a
PVCT 169	"	"	1992	B	0,0 ^a

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^b Antagonistic groups (see also Cirvilleri *et al.*, 2005).

^c Pathogenicity tests performed with *P. s. pv. syringae* strains. The number refers to the mean diameter of lesion recorded seven days after artificial inoculation of lemon cv. Femminello fruits. Mean are based on four replications per experiment. Each experiment was repeated twice. Number in column followed by the same letter are not significantly different using the Student-Newman-Keul's mean separation test at $P \leq 0.05$ (see also Cirvilleri *et al.*, 2005).

beled at 5' end with cy5 fluorophore, 1 μ l of 10 mM each deoxynucleoside triphosphates (dNTPs), and 5 U of *Taq* polymerase. T4 DNA ligase, dNTPs and *Taq* polymerase were from Invitrogen-Life Technologies (Paisley, United Kingdom) and all primers and oligos were from MWG Biotech Inc. (High Point, NC, USA).

All PCR reactions were performed in a DNA thermal cycler (GeneAmp PCR system 9600, Perkin Elmer, Norwalk, Conn., USA) following a previously described protocol (Kassama *et al.*, 2002) with some modifications, i. e.: 60 sec at 94°C, 30 sec at 65°C, and 60 sec at 72°C for one cycle; a 12 cycles touch down PCR with annealing temperature reduced from 65°C by 0.7°C at each cycle; and 25 cycles of 30 sec of denaturation at 94°C, 60 sec of annealing at 56°C, and 60 sec of extension at 72°C. This "touchdown" PCR protocol was used to minimize PCR artifacts.

The AFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA se-

quencer (Beckman & Coulter, Fullerton, CA, USA). A mixture containing 2 μ l of PCR products, diluted 1:20 with Sample Loading Solution (SLS), 30 μ l of SLS, 0.5 μ l of CEQ DNA Size Standard Kit-600 (used to normalize the profile) and one drop of mineral oil were loaded on a capillary electrophoresis system CEQ 8000. The set-up of the CEQ 8000 was done according to the manufacturer's instructions.

The data, displayed as peaks in electropherogram files, were analyzed using the CEQ 8000 analysis software. The fragment sizes were determined by comparison with the internal DNA Size Standard Kit, limiting analysis to fragments between 60 and 700 bp.

Reproducibility of peaks in electropherograms was checked by repeating fAFLP reactions two times on three strains examined in this study. Electropherograms of all fAFLP profiles were visually inspected for polymorphisms, with the presence (1) or absence (0) of fragments from 60 to 700 bp scored in a binary matrix and

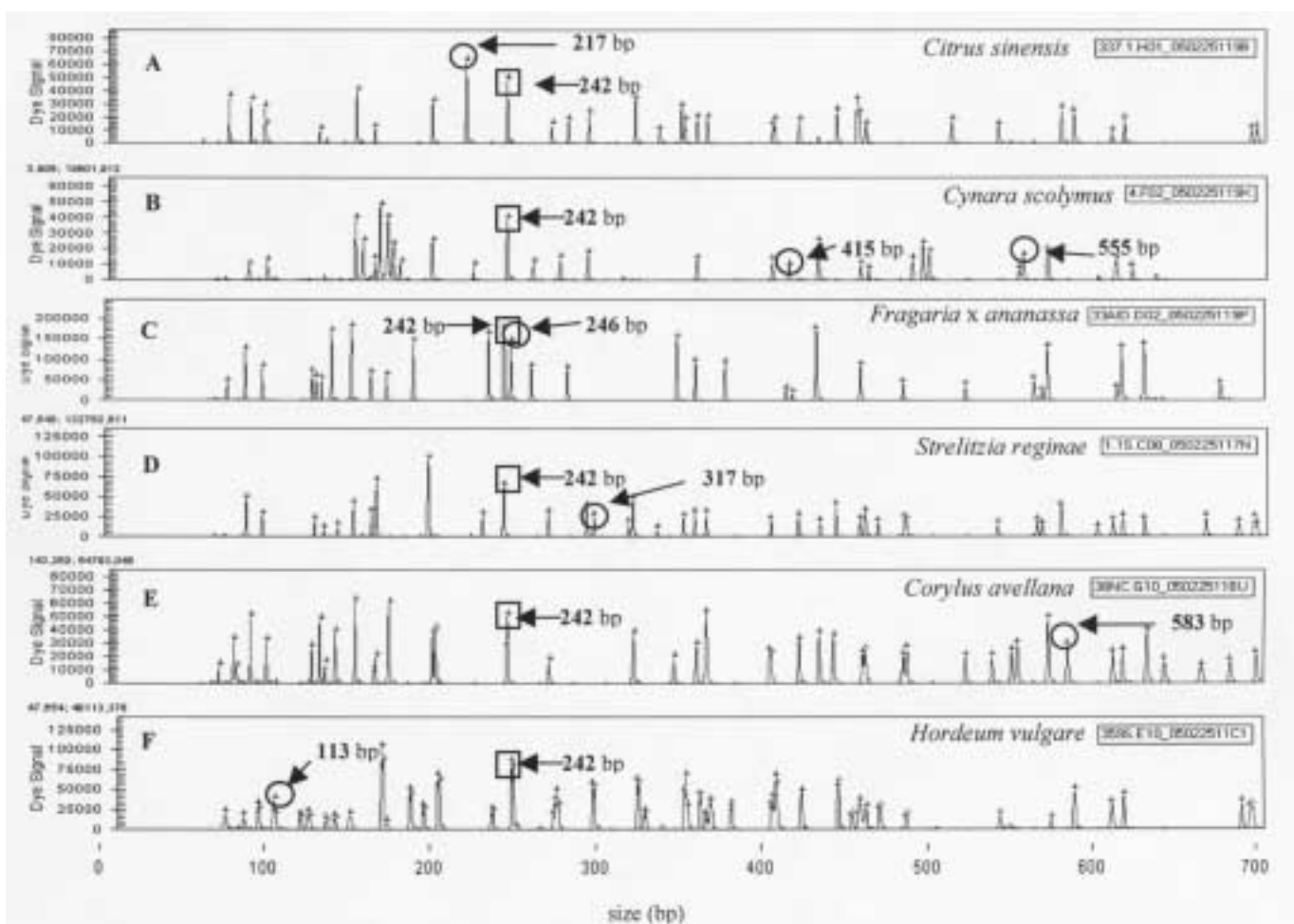


Fig. 1. CEQ 8000 analysis software-derived electropherograms (fAFLP profiles) for *EcoRI*+A plus *MseI*+C amplifications of six representative *Pseudomonas syringae* pv. *syringae* genomes. **A)** PVCT337, isolated from *Citrus sinensis*; **B)** PVCT4 isolated from *Cynara scolymus*; **C)** AID33 isolated from *Fragaria x ananassa*; **D)** PVCT 1.1S isolated from *Strelitzia reginae*; **E)** PVCT33NC isolated from *Corylus avellana*; **F)** ISF359S isolated from *Hordeum vulgare*. Squares indicate the fragment at 242 bp present in most profiles (93% of 57 tested strains); circles indicate fragments typical of strains isolated from the same host. Fragment sizes (expressed as base pairs) were determined by comparison with the standard curve generated from the internal size standard.

stored in Microsoft Excel 2000.

Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) exporting the output files to PHYLIP 3.6 software package (Felsenstein, 2004). Similarity coefficients were determined using the Dice's coefficient (Dice, 1945).

FAFLP analysis showed the presence of 57 different fingerprints. The analysis generated 29 to 43 fragments upon amplification with the primers *MseI*+C and *EcoRI*+A sized within 1 bp.

FAFLP analysis made it possible to identify and characterize strains isolated from the same host, and to distinguish between groups of strains isolated from different hosts. Seven distinct fAFLP clusters linked at a similarity level of 74-95% were identified on the basis of host of origin, although strains isolated from the same host were not necessarily grouped in the same clade (data not shown).

Examples of representative electropherograms are shown in Figure 1. Peak height indicates the relative fluorescence of the detected fragments, and this height did not vary between replicate runs. Fifteen fragments were shared by most of the strains and a 242 bp fragment was common to 93% of the strains. All strains isolated from *Cynara scolymus* showed two specific fragments of 415 and 555 bp, all strains isolated from *Corylus avellana* showed a specific fragment of 583 bp, and all strains isolated from *Strelitzia reginae* showed a specific fragment of 317 bp. In addition, three strains isolated from *Hordeum vulgare* (ISF356, ISF357, ISF359), two from *Fragaria x ananassa* (AID33, AID76) and four from *Citrus sinensis* (PVCT10.2, PVCT337₁, PVCT48SR1, PVCT48SR2), showed specific fragments of 113, 246 and 217 bp, respectively (Fig. 1).

FAFLP analysis was used as a molecular approach to assess the variability of *P. s. pv. syringae* strains isolated from different woody and herbaceous host plants. Variability was observed among isolates from the same host plant as well as among isolates within the same antagonistic group (A and B) or with similar pathogenic activity (Table 1). This variability was in agreement with other investigations performed on the same strains using ERIC-PCR (Cirvilleri *et al.*, 2005) or BOX analysis (Scortichini *et al.*, 2003), or on other strains of *P. s. pv. syringae* using ERIC analysis (Little *et al.*, 1998) or AFLP analysis (Clerc *et al.*, 1998; Manceau and Brin, 2003).

In this study it was found that a dual restriction digest with *EcoRI* and *MseI* as a common primer combination was an excellent approach to generate information-rich fAFLP patterns from all *P. s. pv. syringae* strains. FAFLP profiles revealed that there was no strict correlation between the strains and the host of origin, as previously observed when the same strains were characterized by ERIC-PCR. Clusters of the same *P. s. pv. syringae* strains defined with other previously used methods, such as antagonistic groups and pathogenicity

(Cirvilleri *et al.*, 2005), were not revealed with fAFLP clustering.

Strain-specific discriminative peaks, typically detected in strains isolated from the same host, may be useful for strain identification and for epidemiological studies. On the other hand, the genetic characterization of *P. s. pv. syringae* by fAFLP pointed out an high variability among the strains tested, that is coherent with the occurrence of different populations subjected to varying environmental selection. Although fAFLP seems promising as a reliable technique for specific identification of strains, it was evident that some strains isolated from the same host clustered in separate groups. The discriminatory power of fAFLP (e.g. the number of obtained fragments) can be systematically varied by performing amplification with primers of specified selectivity to produce different numbers and sizes of amplified fragments. Additional data from more strains and additional primer sets are probably needed to optimise the technique.

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REFERENCES

- Boudon S., Manceau C., Notteghem J.L., 2005. Structure and origin of *Xanthomonas arboricola* pv. *pruni* populations causing bacterial spot of stone fruit trees in western Europe. *Phytopathology* **95**: 1081-1088.
- Cirvilleri G., Bonaccorsi A., Scuderi G., Scortichini M., 2005. Potential biological control activity and genetic diversity of *Pseudomonas syringae* pv. *syringae* strains. *Journal of Phytopathology* **153**: 654-666.
- Clerc A., Manceau C., Nesme X., 1998. Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **64**: 1180-1187.
- Dice L.R., 1945. Measures of the amount of ecologic association between species. *Ecology* **26**: 297-302.
- Felsenstein J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6 distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- Grothues D., Rudolph K., 1991. Macrorestriction analysis of plant pathogenic *Pseudomonas* species and pathovars. *FEMS Microbiology* **79**: 83-88.
- Kassama Y., Rooney P.J., Goodacre R., 2002. Fluorescent amplified fragment length polymorphism probabilistic database for identification of bacterial isolates from urinary

- tract infections. *Journal of Clinical Microbiology* **40**: 2795-2800.
- Little E.L., Bostock R.M., Kirkpatrick B.C., 1998. Genetic characterization of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. *Applied and Environmental Microbiology* **64**: 3818-3823.
- Manceau C., Brin C., 2003. Pathovars of *Pseudomonas syringae* are structured in genetic populations allowing the selection of specific markers for their detection in plant samples. In: Iacobellis N.S. et al., (eds). *Pseudomonas syringae* and related pathogens, pp. 503-512. Kluwer Academic Publishers.
- Matarante A., Baruzzi F., Cocconcelli P.S., Morea M., 2004. Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. *Applied and Environmental Microbiology* **70**: 5168-5176.
- Roumagnac P., Gagnevin L., Gardan L., Sutra L., Manceau C., Dickstein E.R., Jones J.B., Rott P., Pruvost O., 2004. Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. *International Journal of Systematic and Evolutionary Microbiology* **54**: 15-24.
- Scholz B.K., Jakobek J.L., Lindgren P.B., 1994. Restriction fragment length polymorphism evidence for genetic homology within a pathovar of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **60**: 1093-1100.
- Scortichini M., Marchesi U., Rossi M.P., Di Prospero P., 2001. Bacteria associated with hazelnut (*Corylus avellana* L.) decline are of two groups: *Pseudomonas avellanae* and strains resembling *P. syringae* pv. *syringae*. *Applied and Environmental Microbiology* **68**: 476-484.
- Scortichini M., Marchesi U., Dettori M.T., Rossi M.P., 2003. Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains from woody and herbaceous host plants. *Plant Pathology* **82**: 277-286.
- Vos P., Hogers R., Bleeker M., Reijans M., Van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kulper M., Zabeau M., 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

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