Phytopathol. Mediterr. (2002) 41, 63–71

Differentiation of *Pseudomonas syringae* subsp. *savastanoi* strains isolated from various host plants by restriction fragment length polymorphism[(1)]

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Summary. A 1.8 kb DNA fragment encoding a putative protein similar to precorrin-3 methylase, a protein of *Pseudomonas aeruginosa* involved in cobalamin biosynthesis, was cloned from an olive strain of the plant pathogenic bacterium *Pseudomonas syringae* subsp. *savastanoi*. This fragment, potentially involved also in virulence, methionine prototrophy and ability to elicit a hypersensitive response, was used as a probe for detecting restriction fragment length polymorphism (RFLP) in 52 strains of *P. s.* subsp. *savastanoi* isolated from olive, oleander and ash. Southern blot analysis revealed a single strongly hybridizing band in all *P. s.* subsp. *savastanoi* strains and *Eco*RI polymorphism was detected among strains isolated from olive plants. In addition, when *Hin*dIII was used as a restriction enzyme, the *P. s.* subsp. *savastanoi* strains isolated from olive clearly differed from those isolated from oleander and ash on the basis of the size of a single hybridizing band. This clear difference between the strains isolated from olive and those isolated from oleander further supports the hypothesis that the populations of *P. s.* subsp. *savastanoi* which infect the above-mentioned host plants differ from one another.

Key words: olive knot bacterium, RFLP, methionine auxotrophy, *cobJ* gene, precorrin-3 methylase.

Abbreviations used in the text: Ap, ampicillin; bp, base pair; Cm, chloramphenicol; DIG, digoxigenin; HR^-, deficient in hypersensitive response; kb, kilobase; KB, King's B medium; Km, kanamycin; *met,* methionine requirement; $Path^-$, altered in pathogenicity; ^{r}, resistant; RFLP, restriction fragment length polymorphism; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; Tn*5*, transposon Tn*5*.

Introduction

Pseudomonas syringae subsp. *savastanoi* (ex Smith, 1908) Janse (1982) and *Pseudomonas savastanoi* pv. *savastanoi* (Gardan *et al.,* 1992) have been reported (Young *et al*., 1996) as synonymous in referring to the plant pathogenic bacterium that causes hyperplastic symptoms on olive (*Olea europaea* L.), oleander (*Nerium oleander* L.) and ash (*Fraxinus excelsior* L.), as well as on other minor host plants. Despite the fact that strains isolated from different host species may be differentiated on the basis of host range and other physiological and pathological characteristics, both of the above-mentioned denominations include all the strains of the pathogen, regardless of their host origin. For example, in the interaction between this pathogen

(1) Dedicated to Professor Antonio Graniti on the occasion of his 75th birthday.

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and olive or oleander, symptom development depends on the ability of the bacterium to produce phytohormones (indole-3-acetic acid and cytokinins) (Surico *et al*., 1985; Surico and Iacobellis, 1992; Iacobellis *et al*., 1994) whereas typical strains isolated from ash do not produce cytokinins and the role of indole-3-acetic acid in symptom development on ash still needs to be demonstrated (Iacobellis *et al*., 1998). In addition, it has been observed that after artificially infecting olive plants with oleander strains, typical hyperplastic symptoms (i.e. knots) form at the inoculation sites, whereas after infecting oleander plants with olive strains necrosis is often induced (Surico *et al*., 1985; Iacobellis *et al*., 1994). On the basis of the differences between strains, also the names *P. savastanoi* pv. *nerii* and *P. savastanoi* pv. *fraxini* were reported as valid alternative names (Young *et al*., 1996) for the strains isolated from oleander and ash respectively. However, the classification of *P. s*. subsp. *savastanoi* is still controversial and unclear because only the denomination *Pseudomonas syringae* subsp. *savastanoi* (= *Pseudomonas savastanoi* pv. *savastanoi*) has been validated for the strains isolated from olive plants.

Strains isolated from olive, oleander and ash have been clustered in different groups using whole cell fatty acid analysis (Janse, 1991) or by analysis of low molecular weight restriction fragments of total DNA (Mugnai *et al*., 1994).

The aim of the present work is to contribute to the molecular characterization of *P. s*. subsp. *savastanoi* strains isolated from different host plants by means of RFLP (restriction fragment length polymorphism) detection. RFLP analysis proved to be useful for the molecular characterization of plants, nematodes, insects (Gabriel and De Feyter, 1992) as well as for differentiating plant pathogenic fungi (Dobinson *et al*., 2000) and bacteria (Lazo *et al*., 1987; Cook *et al*., 1989; Hartung and Civerolo, 1989). Different DNA probes were used for RFLP analysis and, among them, DNA fragments containing genes involved in pathogenicity, hypersensitive reaction elicitation and virulence differentiated the bacterial strains of *P. syringae* pathovars isolated from different host plants (Little and Gilbertson, 1997; Little *et al*., 1998). The cloning of the probe used in this study for detecting RFLP in *P. s*. subsp. *savastanoi* strains is also described in this paper; it is based on previous results (Sisto *et al*., 1999) concerning the identification and characterization of *P. s*. subsp. *savastanoi* mutants induced by transposon (Tn*5*) mutagenesis (Berg, 1989). One of them, named ITM317-69, generated from an olive strain, had a reduced virulence, was markedly hampered in its ability to grow in olive tissues, did not induce any hypersensitive reaction on tobacco and was auxotrophic for methionine. Consistent with the fact that Tn*5* insertion accounted for the above-mentioned mutated phenotypes, a revertant of mutant ITM317-69, which had lost the Tn*5* element from its genome, fully regained all the wild-type features. The DNA fragment cloned from the wild-type strain and used as a probe in the present study corresponds to the DNA regions flanking the Tn*5* element in mutant ITM317-69. Although the characterization of the probe is not required for RFLP analysis, for completeness we also report on the sequencing analysis of the DNA fragment used to differentiate the *P. s*. subsp. *savastanoi* strains and, on the basis of this result, we propose its hypothetical function.

Materials and methods

Bacterial strains, plasmids and culture conditions

Fifty-two *P. s*. subsp. *savastanoi* strains isolated from olive (22 strains), oleander (20 strains) and ash (10 strains) were used in this study (Table 1). Eight of the 22 strains from olive were atypical levan-positive strains isolated in Central Italy (Iacobellis *et al*., 1993). The *P. s*. subsp. *savastanoi* strains were grown at 26°C on King's B agar (King *et al*., 1954) or in King's B broth for 24 h in shaken culture. The *Escherichia coli* strains were grown overnight on LB agar or in LB broth (Sambrook *et al*., 1989) at 37°C. Antibiotics were used at the following concentrations (μg ml^{-1}): ampicillin, 50; kanamycin, 20. Plasmid vector pBluescript SK II (Stratagene, La Jolla, CA, USA) and its recombinant derivatives were propagated in *E. coli* TG1. pCR II-TOPO plasmid vector (Invitrogen, Groeningen, The Netherlands) and its recombinant derivatives were propagated in *E. coli* TOP10 (Invitrogen) (Table 2).

Genetic techniques, DNA manipulations and sequencing

Standard procedures were used for restriction endonuclease digestions, ligations and agarose gel

electrophoresis (Sambrook *et al*., 1989). After gel purification, the DNA fragments were subcloned into digested plasmid vector pBluescript SK II treated with calf intestine alkaline phosphatase. Recombinant plasmids were introduced into *E. coli* TG1 by electroporation as previously described (Sisto *et al*., 1999). The regions flanking the Tn*5* element were sequenced using a single oligonucleotide complementary to and extending outward from the ends of the inverted repeat of the transposon as a primer (Rich and Willis, 1990) and by primer walking. Primers for PCR amplifications and DNA sequencing were designed using ABI Primer Express™ 1.0 software (PE Applied Biosystems, Foster City, CA, USA). In order to amplify the D2 DNA fragment utilized as a probe, the following PCR primers were used: D2-F (5'-TTTTCCGGCACTTTCGAGC-3') and D2-R

Table 1. *Pseudomonas syringae* subsp. *savastanoi* strains used in this study and approximate size (kb) of their *Eco*RI or *Hin*dIII fragments hybridizing to the D2 DNA fragment used as a probe.

Host plant	Strain[a]	Geographical origin	Approximate size of the hybridizing fragment (kb)	
			*Eco*RI	*Hin*dIII
Olive	PVBa229, PVBa230, ITM317, ITM101	Southern Italy (Apulia, Basilicata)	5.7	28
	ITM301, ITM302, ITM304	USA (California)	5.7	28
	PVFi1, PVFi2, PVFi3, PVFi5, PVFi6, PVFi8, PVFiC8, PVFiA14	Central Italy (Tuscany)	3.7	28
	ITMKS1, ITMKL1	Greece	3.7	28
	PVBa206, PVBa207, PVBa224, PVBa227, ITM105	Southern Italy (Apulia, Calabria)	3.7	28
Oleander	ITM305, ITM306	USA (California)	3.7	10.4
	ITM310, ITM311, ITM401, ITM413, ITM516, ITM519, PVBa204, PVBa213, PVBa219	Southern Italy (Apulia, Molise)	3.7	10.4
	ITM601	Northern Italy (Lombardy)	3.7	10.4
	NCPPB640	Yugoslavia	3.7	10.4
	ITM313, ITM315, ITM402, ITM404, ITM521, ITM510	Southern Italy (Apulia, Basilicata, Calabria, Molise)	n.d.	10.4
	ITM602	Northern Italy (Lombardy)	n.d.	10.4
Ash	PVFiF1, PVFiF2, PVFiF3, PVFiF4	Central Italy (Tuscany)	3.7	10.4
	NCPPB1006, NCPPB1464	UK	3.7	10.4
	CFBP1838	France	3.7	10.4
	PD120, PD161, PD179	The Netherlands	3.7	10.4

[a] PVBa, Collection of Istituto di Microbiologia Agraria e Tecnica, Università degli Studi, Bari, Italy; ITM, Collection of Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy; PVFi, Collection of Dipartimento di Biotecnologie Agrarie-Patologia vegetale, Università degli Studi, Firenze, Italy; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; CFBP, Collection Française des Bactéres Phytopathogènes, INRA, Angers, France; PD, Plantenziektenkundige Dienst, Wageningen, the Netherlands.

n.d., not determined.

Table 2. Bacterial strains and plasmids used in this study for cloning the D2 DNA fragment used as a probe.

Strain or plasmid	Relevant characteristics [a]	Source or reference
Escherichia coli		
TG1	*thi*	(Gibson, 1984)
TOP10	*recA1*	Invitrogen (Groeningen, NL)
P. syringae* subsp. *savastanoi		
ITM317	wild-type strain isolated from olive	(Surico *et al*., 1985)
ITM317-69	ITM317R::Tn*5* Rif^r Km^r Sm^r *met* HR^- $Path^-$	(Sisto *et al*., 1999)
Plasmids		
pBR322	Ap^r Tc^r Cm^r	(Bolivar, 1978)
pBluescript SK II	Ap^r	Stratagene (La Jolla, CA)
pCR II-TOPO	Ap^r, Km^r	Invitrogen
pITM-69	11.7-kb *Eco*RI fragment carrying Tn*5* from ITM317-69 cloned into pBR322	(Sisto *et al*., 1999)
pF1	6.0-kb *Eco*RI-*Hin*dIII fragment subcloned from pITM-69 into pBluescript SK II	This study
pF2	2.3-kb *Eco*RI-*Hin*dIII fragment subcloned from pITM-69 into pBluescript SK II	This study
pCR II-D2	1.8-kb D2 fragment amplified from ITM317 and cloned into pCR II – TOPO. This fragment corresponds to DNA regions flanking Tn*5* insertion in mutant ITM317–69	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; ^{r}, resistant; *met,* methionine requirement; *recA1,* defective in recombination; *thi,* thiamine requirement; ITM, Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy; HR^-, deficient in hypersensitive response elicitation; $Path^-$, altered in pathogenicity.

(5'-GCACGCTGCGTCAGTTGTAT-3'). The final concentrations of the components in the PCR reaction mixture were: 1ng μl^{-1} of genomic DNA, 1mM $MgCl_2$, 10mM Tris-HCl, 50mM KCl, 0.5 μM (each) primer, 0.2 mM (each) deoxynucleoside triphosphate (dNTP), 0.03 U μl^{-1} of Taq DNA polymerase. PCR amplifications were performed in a model PE 9700 Perkin-Elmer thermocycler (PE Applied Biosystems) at the following conditions: an initial denaturation at 94°C for 5 min; 30 cycles consisting of 94°C for 45 s, then 55°C for 1 min, extension at 72°C for 2 min; and a single final extension at 72°C for 10 min, followed by cooling at 4°C. The PCR amplified product used as a probe was purified by agarose gel electrophoresis and cloned into pCR II-TOPO plasmid vector (TOPO TA Cloning kit, version C, Invitrogen). DNA sequencing was performed by means of BigDye™ Terminator Chemistry from Perkin-Elmer Applied Biosystems on an ABI 310 sequencer (PE Applied Biosystems). Sequenced DNA fragments were examined for similarity to the deposited sequences using the BLASTN or the BLASTX programs, both made available by the National Centre for Biotechnolo-

gy Information (http://www.ncbi.nlm.nih.gov/). The genomic DNA of *P. s.* subsp. *savastanoi* strains was extracted using the Nucleo Spin Tissue kit (Macherey-Nagel, Duren, Germany). For Southern blot analysis, approximately 1.5 μg of genomic DNA was digested with *Eco*RI or *Hin*dIII restriction enzymes, electrophoresed on 0.7% agarose gels and transferred to a positively charged nylon membrane (Roche Molecular Biochemicals) using a vacuum blotter apparatus (Biorad, CA, USA) under conditions recommended by the manufacturer. DNA labelling, hybridization and detection were carried out using a non-radioactive digoxigenin-labelling and detection kit (Roche Molecular Biochemicals, Monza, Italy) according to the supplier's instructions.

Results

Cloning and sequencing the DNA fragment used as a probe

The cloning of the Tn*5*-containing *Eco*RI fragment from mutant ITM317-69 (Table 2) (Fig. 1) was previously described (Sisto *et al*., 1999) and the recombinant plasmid pITM-69 (Table 2) was obtained. The *Eco*RI-*Hin*dIII fragments from plasmid pITM-69 were individually subcloned into pBluescript plasmid vectors. They were named F1 and F2 and had an approximate size of 6.0 and 2.3 kb respectively (Table 2) (Fig. 1). The DNA flanking Tn*5* insertion within the F2 fragment was sequenced completely, whereas the DNA region adjacent to the transposon within the F1 fragment was only partially sequenced. Database searches for similarities revealed the presence of an IS*51* (Comai and Kosuge, 1983) element at approximately 800 basepairs (bp) from Tn*5* insertion in the F1 fragment. Based on the DNA sequences two PCR primers were designed: one of them (primer D2-R) was 4 bp from the *Eco*RI site of the F2 fragment and the other (primer D2-F) was designed to be situated as close as possible (21 bp) to the IS*51* element within the F1 fragment. From wild-type parental strain ITM317 (Table 2) these primers were expected to amplify a fragment that did not include the IS*51* element; it contained about 800 bp of flanking DNA from one side and 1000 bp from the other side of Tn*5* insertion. This PCR product, with an expected size of about 1.8 kb, was obtained from ITM317 and cloned into pCR-II plasmid vector. It was named fragment D2 and used as a probe in Southern blot experiments. Even though wild-

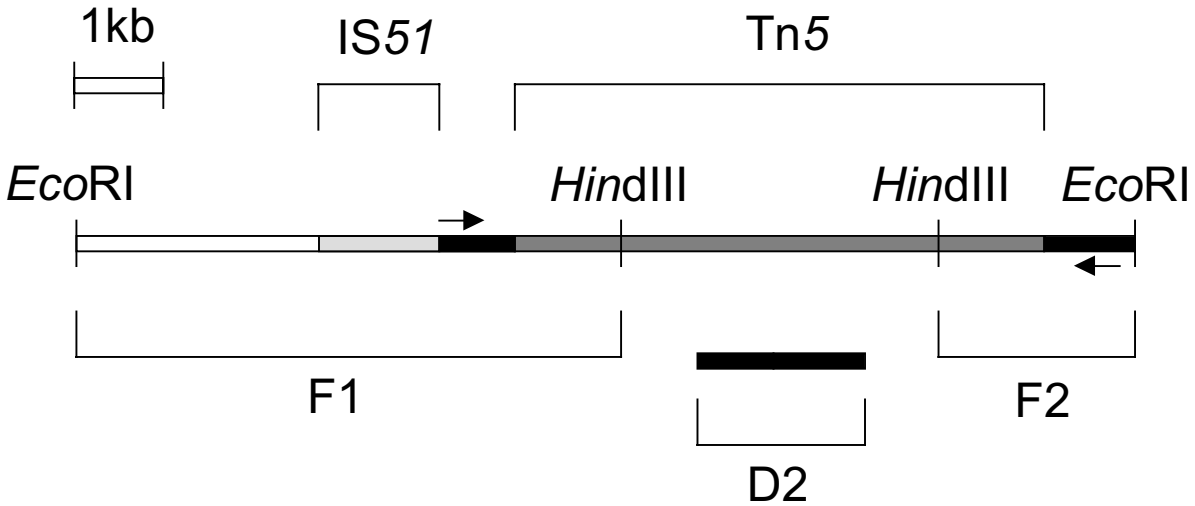

Fig. 1. Map of the *Eco*RI Tn*5*-containing fragment cloned from mutant ITM317-69 of *Pseudomonas syringae* subsp. *savastanoi*. F1 and F2 are 6 kb and 2.3 kb *Eco*RI-*Hin*dIII fragments respectively, subcloned from pITM-69 into pBluescript SK II; Tn*5*, transposon Tn*5*; IS*51*, insertion element IS*51*; D2, DNA fragment corresponding to DNA flanking Tn*5*, cloned from the wild-type strain ITM317 and used as a probe in this study. The black colour denotes the D2 fragment corresponding to DNA flanking Tn*5*; dark grey denotes the Tn*5* transposon; pale grey the IS*51* element. The arrows indicate the primers D2-F and D2-R used to amplify the D2 fragment from the wild-type strain ITM317.

type parental strain ITM317 contained many plasmids (Surico *et al*., 1985), Tn*5* insertion occurred in the chromosome of mutant ITM317-69 (Sisto *et al*., 1999), and as a consequence fragment D2 was also supposed to be cloned from the chromosome of the wild-type strain. Sequence analysis of the D2 fragment confirmed that it was identical to the DNA regions flanking the Tn*5* element in the mutant. In addition, a database search revealed that, spanning from nucleotide 284 to 1792, the predicted protein encoded by the D2 fragment was 66% identical to the protein precorrin-3 methylase of *P. aeruginosa* encoded by the *cobJ* gene and involved in cobalamin biosynthesis (Stover *et al*., 2000).

Southern blot analysis

When a Southern blot of *Eco*RI-digested genomic DNAs from wild-type strain ITM317 and its mutant ITM317-69 was probed with a DIG-labelled D2 fragment, only a single hybridizing band was found in each strain. In ITM317 the hybridizing band had a size of about 5.7 kb; in the mutant it was 5.8 kb longer, consistent with the insertion of the Tn*5* transposon (Fig. 2).

Southern hybridization of *Eco*RI-digested total DNA from all 52 *P. s.* subsp. *savastanoi* strains with the D2 DNA fragment used as a probe revealed only a single strongly hybridizing band in each strain (Table 1). The approximate size of the band was of 3.7 kb in the DNA of all the strains isolated from ash or oleander, whereas a restriction fragment length polymorphism was detected among the strains isolated from olive. In particular, the 4 strains isolated in southern Italy and the 3 strains isolated in California showed a band that strongly hybridized to the probe with an approximate size of 5.7 kb. The other olive strains showed a band of about 3.7 kb, like those isolated from oleander and ash (Table 1, Fig. 3). When *Hin*dIII was used as a restriction enzyme in Southern blot experiments, the *P. s.* subsp. *savastanoi* strains isolated from olive clearly differed from the oleander and ash strains; the former exhibited only one hybridizing band of about 28 kb, whereas the latter showed a hybridizing band of about 10.4 kb (Table 1, Fig. 3). Since, on the basis of these results, *Hin*dIII seemed to be more useful than *Eco*RI to distinguish strains isolated from different host plants, some oleander strains were not further analyzed using the latter restriction enzyme (Table 1).

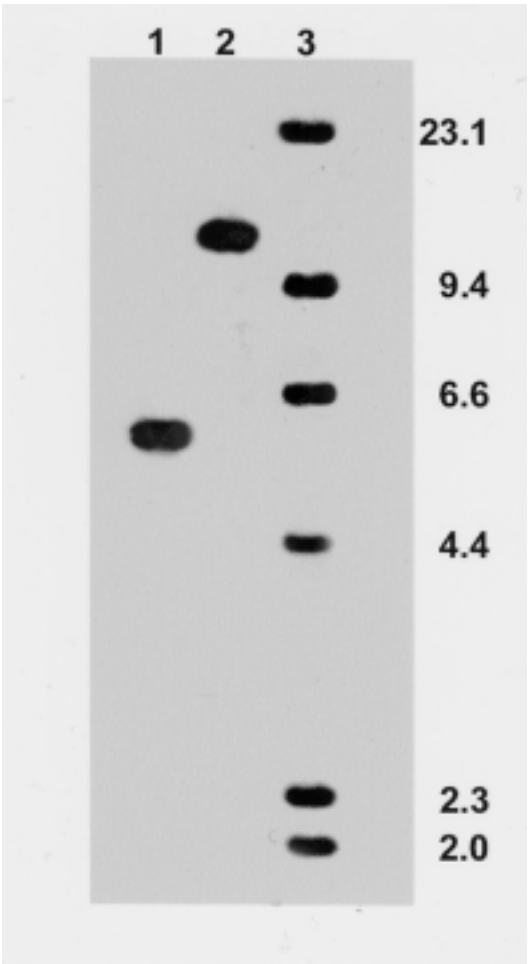

Fig. 2. Southern blot analysis of *Eco*RI-digested genomic DNAs from the wild-type strain ITM317 of *Pseudomonas syringae* subsp. *savastanoi* and its Tn*5*-induced mutant ITM317-69 probed with the D2 DNA fragment. Lanes: 1, wild-type strain ITM317; 2, mutant ITM317-69; 3, DIG-labelled mol. wt marker II (Roche). The numbers (kb) on the right refer to the weight marker.

Discussion

From the olive strain ITM317 of *P. s.* subsp. *savastanoi* we have cloned a 1.8 kb DNA fragment (named D2) that is useful as a molecular probe for differentiating olive strains of *P. s.* subsp. *savastanoi* from the oleander and ash strains of this plant pathogenic bacterium on the basis of the size of a single hybridizing band. The D2 DNA fragment contains a sequence of about 1800 bp that is inter-

rupted by the Tn*5* insertion in the pleiotropic mutant ITM317-69. DNA sequence analysis of the D2 fragment suggests that Tn*5* insertion occurred within a putative open reading frame encoding a protein similar to the enzyme precorrin-3 methylase of *P. aeruginosa*. The latter enzyme, encoded by the *cobJ* gene, is involved in the biosynthesis of cobalamin (vitamin B_{12}), known to be a cofactor of various enzymes. In the case of *P. s.* subsp. *savastanoi*, cobalamin also seems to be required for the altered phenotypes of mutant ITM317-69, i.e. virulence, ability to grow in olive tissues, hypersensitive reaction elicitation and methionine biosynthesis (Sisto *et al.,* 1999). The auxotrophy of mutant ITM317-69 for this amino acid suggests that in *P. s.* subsp. *savastanoi*, as in *Escherichia coli* and other prokaryotes (Fritsch *et al.,* 2000; Kasai and Yamazaki, 2001), methionine biosynthesis involves a cobalamin-dependent methionine synthase (Banerjee and Matthews, 1990) encoded by the *metH* gene. The effect of cobalamin biosynthesis on the other mutated phenotypes can be either direct or indirect. In this regard, it is interesting to note that the ability to synthesize methionine has been reported to be a requirement by other plant pathogenic bacteria for virulence, pathogenicity and/or ability to induce a hypersensitive response in plants (Andersen *et al*., 1998, and cited literature). In addition, it has been suggested that methionine prototrophy plays a role in bacterial stress tolerance and it has been found to be a requirement for the epiphytic fitness of *P. s.* pv. *syringae* (Andersen *et al*., 1998).

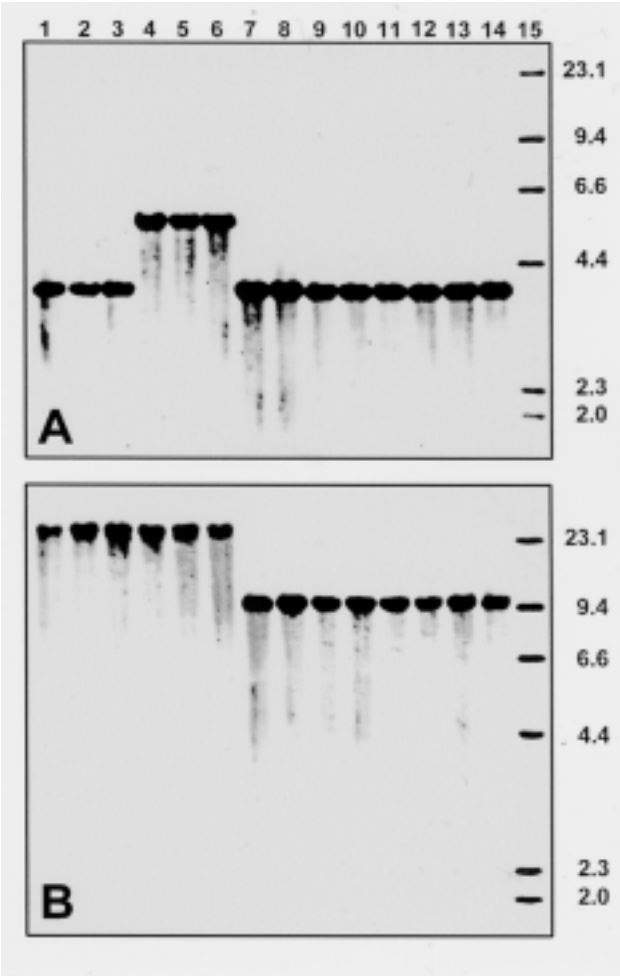

Fig. 3. Southern blot analysis of *Eco*RI-digested (A) or *Hin*dIII-digested (B) genomic DNAs from strains of *Pseudomonas syringae* subsp. *savastanoi* probed with the D2 DNA fragment. Lanes: 1–6, strains isolated from olive (1, PVFiC8; 2, PVFiA14; 3, PVFi8; 4, ITM101; 5, ITM304; 6, PVBa229); 7–11, strains isolated from oleander (7, ITM310; 8, ITM401; 9, ITM413; 10, PVBa219; 11, ITM601); 12–14, strains isolated from ash (12, PVFiF4; 13, PD161; 14, PD120); 15, DIG-labelled mol. wt marker II (Roche). The numbers (kb) on the right refer to the weight marker.

Different kinds of DNA probes have been used for RFLP analysis (Lazo *et al*., 1987; Cook *et al*., 1989; Hartung and Civerolo, 1989; Gabriel and De Feyter, 1992). When large DNA fragments, or DNA fragments containing repeated sequences, are used as probes, detection of RFLP generally results in complex hybridization patterns. These patterns lead to a more complete information about genetic diversity, but they also require further numerical analysis in order that an understanding may be furthered and quantitative data about genetic distance may be obtained (Gabriel and De Feyter, 1992). The D2 DNA fragment used as a probe in this study, was quite small and did not contain sequences that were present in the genome in multiple copies. As a consequence, Southern blot analysis of genomic DNA from all the fifty-two *P. s.* subsp. *savastanoi* strains tested in this study revealed only a single strongly hybridizing band. Although a close relationship between the size of the band and the geographical origin of the strain cannot be established, it is interesting to observe that all the atypical levan-positive non-fluorescent strains isolated from olive in central Italy (Iacobellis *et al*.,

1983) showed an *Eco*RI band of about 3.7 kb, whereas the olive strains isolated in California showed an *Eco*RI band of about 5.7 kb. At present it is unknown whether the different size of the *Eco*RI hybridizing fragments might correlate with other features of the different strains. In addition, using the D2 DNA fragment as a probe and *Hin*dIII as a restriction enzyme in Southern blot experiments, the *P. s.* subsp. *savastanoi* strains isolated from olive clearly differed from oleander and ash strains on the basis of a single hybridizing band related to the host plant. The 28-kb *Hin*dIII fragment seems to be highly conserved in olive strains, as is the 10.4-kb *Hin*dIII fragment in oleander and ash strains, despite their different geographical origins**.** Although it is known that *P. s.* subsp. *savastanoi* strains contain many plasmids (Surico *et al*., 1985), Southern blot experiments were at first carried out in the present study using only total DNA because the probe was cloned from the chromosome of wild-type strain ITM317. Nevertheless, it would be interesting to ascertain, in a future work, whether the different sizes of the hybridizing fragments correlate with their location on a plasmid or on the chromosome. In conclusion, we have identified and cloned from *P. s.* subsp. *savastanoi* a DNA fragment potentially involved in cobalamin biosynthesis which also seems to be involved, either directly or indirectly, in virulence, the ability to grow in olive tissues, hypersensitive reaction elicitation and methionine biosynthesis. This fragment is useful as a molecular probe for differentiating olive strains of *P. s.* subsp. *savastanoi* from oleander and ash strains on the basis of the size of a single hybridizing band. This clear difference between the strains isolated from olive and those isolated from oleander further supports the results of the study by Caponero *et al*. (1995), suggesting that the populations of *P. s.* subsp. *savastanoi* which infect the above-mentioned host plants differ from one another and that, under natural conditions, oleander-strains do not infect olive trees.

In addition, the restriction fragment length polymorphism detected among *P. s.* subsp. *savastanoi* strains indicates and confirms (Mugnai *et al*., 1994) that molecular markers, besides other physiological and pathological features, can be used to differentiate the *P. s.* subsp. *savastanoi* strains isolated from olive from those isolated from other host plants.

Acknowledgements

We thank Prof. G. Surico for providing several *P. s.* subsp. *savastanoi* strains. We also thank Mr. G. Stea for his technical assistance in DNA sequencing and Mr. F. De Marzo for processing the photographs. This work was financially supported by a joint MURST/CNR Italian program within the FESR Project "*Interventi per la salvaguardia e la valorizzazione della biodiversità*", coordinated by Dr. E. Desiderio.

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Accepted for publication: March 12, 2002