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Multiphasic approach for the identification of the different classification levels of *Pseudomonas savastanoi* pv. *phaseolicola*

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Abstract

The relationships between strains of *Pseudomonas savastanoi* pv. *phaseolicola* (*P. sav. phaseolicola*), *P. syringae* pv. *tabaci* (*P. syr. tabaci*) and *P. syr. syringae* which all cause disease on bean; the related species *P. sav. glycinea* and *P. syr. actinidiae*, and reference bacteria, were evaluated by studying the phenotypic and genetic diversity of a collection of 62 strains. All the *P. sav. phaseolicola* strains tested produced characteristic watersoaked lesions on bean pods. Other pathovars produced varying combinations of symptoms including necrotic lesions, with or without watersoaked centres and sunken tissue collapse of the lesion (*P. syr. tabaci*) and necrotic lesions with or without sunken collapse (*P. syr. syringae*). At the genomospecies level, all the strains of *P. sav. phaseolicola*, *P. sav. glycinea* and *P. syr. tabaci*, belonging to genomospecies 2, could be separated from *P. syr. syringae* strains (genomospecies 1) and *P. syr. actinidiae* strains (unknown genomospecies) by BOX-PCR and DNA/DNA hybridisation. To distinguish *P. sav. phaseolicola*, within genomospecies 2, from *P. sav. glycinea* and *P. syr. tabaci*, it was necessary to perform nutritional characterisations (myo-inositol negative and p-hydroxy benzoate positive for *P. sav. phaseolicola* strains), PCR with specific primers designed from the *tox* region (positive for all of the *P. sav. phaseolicola* strains) and serotyping, as 71% of the *P. sav. phaseolicola* strains reacted as O-serogroup PHA1. Important intrapathovar variation was seen by genomic fingerprinting with REP and ERIC primers, as well as with RAPD primers (AE7 and AE10) and esterase profilings. While RAPD fingerprinting detected variability correlated with two race-associated evolutionary lines, REP, ERIC and esterase profiles revealed intrapathovar variation linked to some host origins, that separated the kudzu isolates, and the mungbean isolates, from the other *P. sav. phaseolicola* strains.

Introduction

Beans (*Phaseolus vulgaris* L.) may suffer from various bacterioses incited by pseudomonads, according to their geographical and climatic growth conditions. Brown spot disease, due to *Pseudomonas syringae* pv. *syringae*, occurs in the U.S.A., Northern Europe and Australia (Rudolph, 1979; Cheng et al., 1989). Bean wildfire, due to *P. syringae* pv. *tabaci*, is only reported in tropical countries (Ribeiro et al., 1978). Halo blight, due to *P. savastanoi* pv. *phaseolicola*, shows a wide distribution since it affects bean in temperate areas as well as tropical highlands. This latter bacterium has a large host range within the *Phaseolae* tribe of legumes, including *Phaseolus* spp. (*P. vulgaris, P. coccineus* and *P. lunatus*), *Vigna* spp . *(V. radiata, V. unguiculata*), and a series of weed and wild plant species among which is the kudzu vine (*Pueraria lobata*) (Goto et al., 1981; Taylor et al., 1996). A pathovar may be subdivided into pathogenic races, groups of strains that are virulent

only on some reference cultivars of their host plant. For instance, nine races were described within *P. savastanoi* pv. *phaseolicola* (Taylor et al., 1996).

The taxonomy of *P. syringae* (LOPAT I of Lelliott et al., 1966) is currently under revision, since nine genomospecies were recently described (Gardan et al., 1999). However, all genomospecies cannot reliably be distinguished by techniques other than quantitative DNA : DNA hybridisation which is not suitable for routine diagnosis. Unequivocal identification of a pathovar requires host plant inoculation, and races within a pathovar can be distinguished only on the basis of differential cultivar inoculation. New specific genomic fingerprinting has been proposed as a diagnostic tool (Versalovic et al., 1994; Rademaker and Bruijn, 1997; Rademaker et al., 1998) and involves amplification of interspersed repetitive DNA sequences present in bacterial genomes, referred to as rep-PCR (Rademaker and Bruijn, 1997) or amplification of random sequences by arbitrary primers (Williams et al., 1990; Welsh and McClelland, 1990). This approach was useful for the delineation of species (Onfroy et al., 1999), subspecies (Louws et al., 1998) or pathovars (Louws et al., 1994; Völksch and Weingart, 1997) in some instances.

The present study aims to clarify the relationships between *P. syringae* pv. *syringae*, *P. syringae* pv. *tabaci* and *P. savastanoi* pv. *phaseolicola* at the species, pathovar and race levels.

Materials and methods

Bacterial strains

Phytopathogenic fluorescent pseudomonads belonging to the *Pseudomonas syringae* group (Palleroni, 1984) were obtained from the 'Collection Française des Bactéries Phytopathogènes' (CFBP, Angers, France), and comprised 62 strains (Table 1): 41 strains of *P. savastanoi* pv. *phaseolicola*, including representatives of the nine races of the bacteria, isolated from a wide range of hosts and geographical origins (Taylor et al., 1996); 11 representative strains of two other bacteria pathogenic to bean (*P. syringae* pvs. *tabaci* and *syringae*); four strains of the very closely related *P. savastanoi* pv. *glycinea*; four strains of *P. syringae* pvs. *syringae* and *actinidiae* because of their capability of producing phaseolotoxin (Tamura et al., 1989; Tourte and Manceau, 1995); and two strains, 3650 and 3662, received as *P. savastanoi* pv. *phaseolicola*, but which differed considerably and are listed separately as unknown pathovars. In the following text, the ternary nomenclature will be designated by the abreviation *P. syr. syringae* instead of *P. syringae* pv. *syringae*.

Pathogenicity tests

(a) On bean pods. Pathogenicity tests were performed on bean pods of cv. 'Michelet', which is susceptible to all races of *P. sav*. *phaseolicola*, grown in the greenhouse, and selected at the early stages of seed formation. Excised pods were sterilised with chlorine water, inoculated by infiltrating a suspension of 10^8 cfu ml^{-1} distilled water, under the epidermis (Klement and Lovrekovich, 1961). Nine pods per strain were placed on water agar (1% w v^{-1}) in large glass Petri dishes. The pods were incubated in a controlled environment chamber (20 °C, 14 h daylight), and reactions were recorded between 5 and 7 days.

(b) On bean and tobacco plants. Potted bean plants, cv. 'Michelet', grown in the greenhouse at the stage of four trifoliate leaves were inoculated with *P. sav. phaseolicola* and *P. syr. tabaci*. Bacterial suspensions ($3x10^8$ cfu ml^{-1} in distilled water) were sprayed on to the bean leaves. The fourth leaf was infiltrated under high pressure by the sprayer, applied 1 cm from the lower surface of the leaf, then the whole plant was gently sprayed with the suspension. The inoculated plants were covered for 3 days with a plastic bag, which acted as a humidity chamber, in the greenhouse. Symptoms were recorded 6–8 days after inoculation. Tobacco plants, cv. 'Xanthi', were also inoculated under pressure with *P. syr. tabaci* strains.

Nutritional characterisation

Assimilation of 99 carbon sources (sugars, alcohols, amino acids and organic acids) was performed with the Biotype 100 system (BioMérieux, La Balme-les-Grottes, France). The strips were inoculated with Biotype Medium 1 as recommended by the manufacturer, and the results read visually at two, four and six days after incubation at 28 °C. In addition, 19 conventional biochemical tests were carried out: arginine dihydrolase, oxidase, gelatin, nitrate reduction, levan, fluorescence, esculin, pectinolysis on calcium pectinate, Tween esterase, DNAse, polypectate hydrolysis at pH 5 and 8.3, and the utilisation of sucrose, lactate, L(+)tartrate, D(−)tartrate, erythritol, mannitol,

Table 1. Origin and characteristics of the 62 strains of *Pseudomonas savastanoi* pv. *phaseolicola*, *Pseudomonas syringae* pvs. *syringae* and *tabaci* isolated from bean and related species *P. savastanoi* pv. *glycinea* and *P. syringae* pv. *actinidiae*

CFBP[1] Nr	Year of isolation, country, author's designation	Host	Interest	Path test[2]	PCR[3]	Serology	
						O-serogroup[4]	Flagella[5]
P. syringae pv. *syringae* (Genomospecies 1)[6]							
1392[T]	1950, UK, Sabet NCPPB 281	*Syringa vulgaris*	Pathotype	C	–	PERSAVTOM1	H1
3388	1992, France, Manceau Psi3	*Vicia sativa*		C	+	MOP2	H1
4886	1985, USA, Hirano 5B530	*Phaseolus vulgaris*		C	–	MOP4	H1
4887	1990, USA, Hirano 9b1	*P. vulgaris*		D	–	nt[7]	H1
4888	1981, USA, Hirano B728a	*P. vulgaris*		C	–	nt	H1
P. savastanoi pv. *glycinea* (Genomospecies 2)							
2214	1968, NZ, Watson, B 149	*Glycine max*	Pathotype	C	–	APTPIS	H2
3356	1984, USA Stall R0	*G. max*	Race 0	E	–	HEL2	nd[8]
3357	1984, USA, Stall R1	*G. max*	Race 1	D	–	APTPIS	nd
3361	USA, Fett A29-2	*G. max*	Race 4	C	–	HEL2	H2
P. savastanoi pv. *phaseolicola* (Genomospecies 2)							
1390*[9]	1949, Canada, NCPPB 52	*P. vulgaris*	Pathotype (Race 5)	nd	+	PHA1	H2
3632	1984, UK, Taylor 1281A	*P. coccineus*	Race 1	A	+	PHA1	H2
3633*	1985, UK, Taylor 1474A	*P. coccineus*	Race 1	nd	+	PHA1	nd
3634	1988, Italy, Taylor 1996A	*P. coccineus*	Race 1	nd	+	PHA1	nd
3635	1975, USA, Taylor 882	*P. vulgaris*	Race 2	A	+	PHA1	nd
3636*	1972, Australia, Taylor 1650	*Macroptilium atropurpureum*	Race 2	nd	+	PHA1	nd
3637	1987, Yemen, Taylor 1839A	*P. vulgaris*	Race 2	nd	+	PHA1	nd
3638*	1984, Tanzania, Taylor 1310A	*P. vulgaris*	Race 3	A	+	PHA1	nd
3639	1985, Tanzania, Taylor 1427B	*P. vulgaris*	Race 3	nd	+	PHA1	H2
3640	1989, Colombia,Taylor 2475A	*P. vulgaris*	Race 3	nd	+	PHA1	nd
3641*	1984, Rwanda, Taylor 1302A	*P. vulgaris*	Race 4	A	+	PHA1	nd
3642	1985, Burundi, Taylor 1385A	*P. vulgaris*	Race 4	nd	+	PHA1	nd
3643	1985, Rwanda,Taylor 1499A	*P. vulgaris*	Race 4	nd	+	PHA1	nd
3644*	1985, Kenya, Taylor 1375A	*Lablab purpureus*	Race 5	A	+	MOP3	nd
3645*	1986, Tanzania, Taylor 1516A	*Desmodium* sp.	Race 5	nd	+	MOP3	nd
3646*	1990, Malawi, Taylor 2708A	*P. vulgaris*	Race 5	nd	+	PHA1	nd
3647*	1984, Tanzania, Taylor 1299A	*P. vulgaris*	Race 6	A	+	PHA1	nd
3648	1989, Lesotho, Taylor 2368A	*P. vulgaris*	Race 6	nd	+	PHA1	nd
3649	1990, Zimbabwe, Taylor 2694A	*P. vulgaris*	Race 6	nd	+	PHA1	nd
3651*	1985, Ethiopia, Taylor 1449B	*L. purpureus*	Race 7	A	+	PHA1	nd
3652*	1986, Tanzania, Taylor 1537C	*Vigna radiata*	Race 7	nd	+	APTPIS	nd
3653	1986, Tanzania, Taylor 1542A	*Neonotonia wightii*	Race 7	nd	+	MOP3	nd
3654*	1971, USA, Taylor 1644	*V. radiata*	Race 1	nd	+	APTPIS	nd
3655	1970, NZ, Taylor 1674A	*V. angularis*	Race 7	nd	+	PHA1	nd
3656*	1964, Tanzania, Taylor 1645	*Dolichos* sp.	Race 8	nd	+	PHA1	nd
3657*	1990, Lesotho, Taylor 2656A	*P. vulgaris*	Race 8	A	+	PHA1	nd
3658	1990, Lesotho, Taylor 2659A	*P. vulgaris*	Race 8	nd	+	PHA1	nd
3660*	1990, Malawi, Taylor 2709A	*P. vulgaris*	Race 9	A	+	PHA1	nd
3661	1990, Colombia, Taylor 2732E	*P. vulgaris*	Race 9	nd	+	MOP3	nd
3663*	USA, Taylor 3012	*V. radiata*	Race 1	A	+	APTPIS	H2
4704	1993, France (?), Olivier 42120	*P. vulgaris*	nt	A	+	PHA1	nd
4705	1997, Olivier 100197	*P. vulgaris*	nt	A	+	PHA1	nd
4706	France, Fouilloux Brion	*P. vulgaris*	nt	A	+	PHA1	nd
4847	1997, France, Samson SR533-4	*P. vulgaris*	Race 6	A	+	PHA1	nd
4848	1997, France, Samson SR640-9	*P. vulgaris*	nt	A	+	PHA1	nd

Table 1. (*Continued*)

CFBP[1] Nr	Year of isolation, country, author's designation	Host	Interest	Path test[2]	PCR[3]	Serology	
						O-serogroup[4]	Flagella[5]
4849	1997, France, Samson SR641-2	*P. vulgaris*	Race 7	A	+	PHA1	nd
4850	USA, KH1a1	*Pueraria lobata*	Race 7	A	+	PHA2	H2
4851	USA, KW1b1	*P. lobata*	Race 7	A	+	RIB	H2
4852	Japan, KZ1H	*P. lobata*	Race 7	A	+	PHA2	H2
4859	Japan, Sato Kuz1	*P. lobata*	nt	A	+	PHA2	H2
4860	Japan, Sato Kuz4	*P. lobata*	nt	A	+	PHA2	H2
P. syringae pv. *tabaci* (Genomospecies 2)							
2106	1959, Hungary, Klement H59	*Nicotiana tabacum*	Pathotype	C	−	TAB	H2
4854	1989, Brazil, Rodrigues N. IBSBF 703	*P. vulgaris*		B	−	TAB	H2
4855	1973, Brazil, Rodrigues N. IBSBF 761	*P. vulgaris*		B	−	TAB	H2
4856	1973, Brazil, Rodrigues N. IBSBF 762	*P. vulgaris*		D	−	TAB	H2
4857	1992, Brazil, Ito IAC 6900	*P. vulgaris*		D	−	RIB	H2
4858	1992, Brazil, Tanaka IAC6528	*P. vulgaris*		B	−	TAB	H2
P. syringae pv. *tabaci* (uncertain pathovar)							
4861	1978, Brazil, Takatsu UnB 13	*P. vulgaris*		D	−	PHA1	H2
P. syringae pv. *actinidiae* (unknown genomospecies)							
10771	Japan, PA 429			D	−	PHA1	H2
4909	1984, Japan, Kw-11	*Actinidia deliciosa*	Pathotype	C	+	PHA1	H2
4911	1984, Japan, ICMP 9854	*A. deliciosa*		C	+	HEL2	H2
P. syringae (unknown genomospecies and pathovar)							
3650∗	1985, Kenya, Taylor 1354A	*P. vulgaris*		E	−	SYR1	H2
3662∗	Tanzania, Taylor 1646	*V. radiata*		D	−	SYR1	H2

[1]Collection Française de Bactéries Phytopathogènes.
[2]Pathogenicity test: Inoculation of a bacterial suspension at 10^8 cfu/ml on 'Michelet' excised bean pods (A = watersoaked green lesion; B = brown lesion with watersoaked centre; C = sunken brown lesion; D = brown lesion; E = no lesion).
[3]PCR: Standard PCR with the specific primers PHA19 and PHA95 and a 480 bp amplified fragment.
[4]O-Serogroups defined by Saunier et al., 1996.
[5]Flagellar serotype defined by Guillorit-Rondeau et al. (1996) and Malandrin and Samson (1999).
[6]Genomospecies designated after Gardan et al. (1999).
[7]Not typed by any of the 23 O-serogroups, or untypable due to weak responses on host differential series.
[8]Not done.
[9]Strains used for quantitative DNA hybridisation.

and sorbitol in ARJ medium (Lelliott and Stead,1987; Gardan et al., 1999). The ability of tobaco to produce a hypersensitive reaction (HR) was also tested.

Serology

Antisera were produced by injecting whole-cell bacteria intravenously in rabbits. O-serogroups were determined according to Saunier et al. (1996). LPS serological reactions were revealed by Ouchterlony double-diffusion tests, using very dense bacterial suspensions in distilled water as antigens. LPS-antibody precipitate appeared as a unique band showing total or partial identity with serological references. Sidechain devoid LPSs were recognized by a specific antiserum delineating the O-serogroup RIB (Saunier et al., 1996). Flagella serotyping was performed by immunofluorescent staining with six antisera (Guillorit-Rondeau et al., 1996).

Phaseolotoxin bio-assay

Phaseolotoxin production was tested by the *Escherichia coli* bioassay (Jansing and Rudolph, 1990).

Esterase isozyme profiling

Bacterial extracts were prepared from cells grown on King's medium B for 48 h. The cells were suspended in an extraction buffer (Tris 10 mM, EDTA 1 mM, 2-mercaptoethanol 3 mM, cysteine 0.002% w v^{-1}, pH 7.0) containing 25 mg ml^{-1} Polyvinylpyrrolidone and sonicated in an ice-bath (Malandrin and Samson, 1998). Protein separation was performed by native vertical PAGE. Esterase bands were revealed by staining the gel with Fast blue RR salt (1 mg ml^{-1}) and α- and β-naphtyl acetates (0.04% w v^{-1}) at 37 °C for 20–30 min, and an image was captured with a CCD camera.

Bacterial cultures and genomic DNA preparation

Strains were grown at 26–28 °C on King's Medium B for 24 h. From these cultures, cells were washed with sterile distilled water and a suspension prepared, which was adjusted to an OD_{560} of 0.2. Aliquots of 500 µl in 2 ml cryotubes were stored at −20°C. For utilisation, after liquefying the suspension at room temperature, cells were lysed for 10 min in a boiling water bath, and the cryotubes kept on ice before use.

Total genomic DNA was extracted using the Rapid-Prep Micro Genomic DNA Isolation Kit for Cells and Tissue (Pharmacia Biotech). The extracted DNA was resuspended in 30 µl of sterile double-distilled pure water, and stored at -20 °C until use.

RAPD and rep-PCR primers

Two 10-mer oligonucleotides (Bioprobe Systems/ Quantum, France) were used to generate randomly amplified polymorphic DNA (RAPD) profiles. Selected primers were chosen based on their performance and capacity to discriminate between bacterial strains, after screening a set of ten primers by visual examination of the generated profiles. The primer sequences were (5′–3′): AE7: GTGTCAGTGG and AE10: CTGAAGCGCA. The rep-PCR primers were: REP1R-I and REP2-I for REP-PCR; ERIC1R and ERIC2 for ERIC-PCR (Versalovic et al., 1991) and BOXA1R for BOX-PCR (Versalovic et al., 1994).

RAPD and rep-PCR analysis

Amplification reactions were performed in volumes of 25 µl, containing 2 µM of a single RAPD or BOX primer, or 50 pM of each REP or ERIC primers; 200 µM each of dATP, dCTP, dGTP and dTTP (Bioprobe Systems/Quantum, France), PCR reaction buffer (10 mM TrisHCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% TritonX100 and 0.2 mg ml^{-1} bovine serum albumin), 1.5 units of *Taq* DNA polymerase (Appligene-Oncor, France) and, as template DNA, 5 µl of a bacterial cell suspension at $10^7 - 10^8$ cfu ml^{-1} for RAPD reactions and 10^8 cfu ml^{-1} for rep-PCR reactions. The premix was prepared for no more than six samples at once for RAPD reactions. For rep-PCR, the premix was prepared for all the samples included in each assay. Amplification was performed in an MJ Research, Inc. PTC-100 Thermal Cycler programmed for an initial denaturation step of 5 min at 95 °C, followed by 45 cycles of 1 min at 95 °C, 1 min at 37 °C and 1 min 30 s at 72 °C with a final elongation step of 15 min at 72 °C for RAPD reactions (Williams et al., 1990; Khan et al., 1999). For rep-PCR amplifications the initial denaturation step of 5 min (7 min for BOX) at 95 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 40 °C, 52 °C or 53 °C, for REP, ERIC and BOX primers respectively, and 5 min (8 min for BOX) at 65 °C with a final elongation step of 15 min at 65 °C.

PCR amplification products were detected by electrophoresis of 12 µl aliquots through 1.4% agarose gels in Tris–borate–EDTA (TBE) buffer (Sambrook et al., 1989), which were stained with ethidium bromide (EtBr 1.25 mg/l), visualised under UV light, and printed image through Bio-Print (Vilber Lourmat, France). DNA standards (1-kb DNA ladder Gibco BRL) were included in each electrophoresis gel.

All the amplifications were performed at least twice in separate assays, to ensure the reproductibility of the patterns, and only bands common to the replicate amplifications were scored. DNA fingerprints of strains were first compared for similarity by visual inspection of band patterns. They were considered identical when all scored bands in each pattern had the same apparent migration distance, even if a slightly different molecular-weight was assigned to the same band over two or three different electrophoreses. Variations in intensity were not taken as differences.

Following the visual inspection, the patterns of all of the isolates were analysed more rigorously by using

the Bio-Profil software (Vilber Lourmat, France). Band sizes were assigned by direct comparison to concurrently run DNA standards (1 kb). This information was used to construct a matrix table where each isolate was matched with a notation ±, where (+) represents the identical presence and position of a band in the fingerprints to be compared.

Data analysis

All dendrograms were established using TAXONUM, a software developed by G. Hunault and L. Gardan (Faculté des Sciences d'Angers, Angers, France). Cluster analysis was carried out using the unweighted pair-group method with averages (UPGMA) with the complement of Jaccard's similarity coefficient. Each RAPD and rep-PCR fragment was considered as a separate marker in pairwise comparisons.

The BOX fragments as well as the biochemical tests characteristic of each cluster were identified by assessing the amount of information provided by each fragment or character, obtained by calculating the diagnostic ability coefficient (DAC) (Descamps and Véron, 1981).

PCR with specific primers

PCR reactions were carried out by using the following specific primers to *P. sav. phaseolicola*, selected from the *tox* region (C. Tourte and C. Manceau, unpub.): PHA19: 5′CGTCTGTAACCAGTTGATCC3′ and PHA95: 5′GAATCCTTGAATGCGAAGGC3′. PHA19 and PHA95 primers amplified a 480 bp DNA fragment of the phaseolotoxin gene cluster (Tourte, 1993). The amplifications were performed in a total volume of 50 µl by mixing 0.6 µM of each primer, 1.2 mM each of dATP, dCTP, dGTP and dTTP (Eurogentec, Belgium), PCR reaction buffer (75 mM Tris–HCl (pH 9.0 at 25 °C), 20 mM $(NH_4)_2SO_4$ 0.1% (w/v) Tween 20, 3.75 mM $MgCl_2$), 1.5 units of *Taq* DNA polymerase (Eurogentec, Belgium) and as template DNA, 10 µl of a 10 min boiled, bacterial-cell suspension at 10^7 cfu ml^{-1}. The same thermocycler described above was used with the following profile: an initial denaturation step of 2 min at 94 °C, followed by 35 cycles of 30 s at 92 °C, 45 s at 52 °C and 1 min 30 s at 72 °C with a final elongation step of 2 min at 72 °C. PCR amplification products were detected by electrophoresis of 10 µl aliquots through 1.5% agarose gels in TBE buffer, and stained with ethidium bromide, visualised under UV light, and image printed as described above. DNA standards (1-kb DNA ladder Gibco BRL) were included in each electrophoresis gel.

Quantitative DNA hybridisation

Bacterial DNA was extracted and purified according to Brenner et al. (1982). Native DNA was labeled *in vitro* by random priming using Megaprime DNA labeling system and ^{3}H-labeled nucleotides (Amersham Int., Amersham, UK). The S1 nuclease and trichloroacetic method was used for DNA–DNA hybridisation, with a reassociation procedure at 70 °C (Crosa et al., 1973; Grimont, 1988). DNA–DNA hybridisation tests were carried out by using labeled DNA from the reference strain of *P. sav. phaseolicola* (CFBP 1390), that belongs to the genomospecies 2 (Gardan et al., 1999), and unlabeled DNAs of a selection of bean strains.

Results

Pathogenicity characteristics and PCR recognition by phaseolotoxin gene primers

A selection of 42 strains was evaluated for pathogenicity on bean plants and/or excised pods. Pathogenicity tests performed on excised bean pods resulted in several types of symptoms (Table 1). The 20 *P. sav. phaseolicola* strains were the only strains to induce the watersoaked lesions (class A symptoms). *P. syringae* strains 3650 and 3662 gave reactions E and D. However, when inoculated on potted bean plants, they induced the formation of numerous brown and watersoaked pin points on sprayed young leaves, as did the other *P. sav. phaseolicola* strains which induced no halo formation in the same inoculation assay. The symptoms produced on beans by *P. syr. tabaci* bean isolates consisted of pin points too, but they were surrounded by an intense chlorotic halo of 3 mm diameter, except for CFBP 4861. On tobacco the same *P. syr. tabaci* strains induced a large chlorotic halo beyond the collapse induced in the infiltrated area.

Positive reactions were obtained for the 41 pv. *phaseolicola* strains, with standard PCR using the specific primers PHA19 and PHA95 (Table 1). *P. syringae* strains CFBP 3650 and 3662 were negative. On the other hand, strains 3388 of *P. syr. syringae*

and two of three strains belonging to *P. syr. actinidiae* (CFBP 4909 and 4911) showed positive reactions. The phaseolotoxin bioassay confirmed that the strains 3388, 4909 and 4911 were toxin producers whereas strain 10771, as well as 3650 and 3662, remained negative after three repeats.

Nutritional characteristics

All the 62 bacterial strains (Table 1) fitted with the general characteristics of *P. syringae sensu lato*. They were obligate aerobes, produced fluorescent pigment on King's medium B (except for *P. syr. actinidiae* strains, *P. sav. glycinea* strain 3356, and *P. sav. phaseolicola* strains 3653 and 4706) and belonged to LOPAT group I of Lelliott et al. (1966).

A numerical analysis of the 119 nutritional characteristics was performed. The dendrogram displaying the distance relationships between the strains is shown in Figure 1. At a distance of 0.33, aiming to group the *P. sav*. *phaseolicola* strains together, the 62 strains studied were clustered in two phena and two isolated strains, *P. sav. glycinea* CFBP 3357 and *P. syr. syringae* 1392^{T}. The phenon A was made up of 45 strains: 41 *P. sav. phaseolicola* strains, three *P. sav. glycinea* strains, and the uncertain *P. syr. tabaci* strain. Phenon B included 15 strains: six *P. syr. tabaci* strains (from bean and tobacco), four *P. syr. syringae* strains (from bean and *Vicia* sp.), the three strains of *P. syr.actinidiae*, and the two unknown pathovar strains 3650 and 3662. At the distance level of 0.14, aiming to separate any *P. sav. phaseolicola* strain from the other pathovars, phenon A was divided into five subphena and 10 isolated strains. The subphenon A1 clustered 27 *P. sav. phaseolicola* strains out of 41. The phena A2, A3, A4 and A5 clustered two *P. sav. phaseolicola* strains each, and eight strains remained isolated. The three *P. sav. glycinea* strains of phenon A did not cluster together.

The most discriminating carbon sources selected from DAC calculation for the phena and the isolated strains are given in Table 2. The two clusters A and B were distinguishable by two substrates only: sorbitol and meso-tartrate. Among the discriminating substrates inside phenon A, myo-inositol was consistently negative for *P. sav. phaseolicola* strains, whereas p-hydroxy-benzoate was consistently positive. The main varying characters for pv. *phaseolicola* were D(+)arabitol (73% negative), D(−)mannitol (71% negative), and dl-glucuronate (80% positive).

Serological characterisation

Serological characterisation of the 62 strains was performed on both types of antigens, cell wall LPS and flagella (Table 1). All *P. syr. syringae* strains exhibited the H1 flagella whereas *P. sav. glycinea, P. sav. phaseolicola, P. syr. tabaci* and *P. syr. actinidiae* exhibited the H2 flagella.

O-serogroups were assigned to all of the 62 strains studied, except two *P. syr. syringae* strains (bean isolates CFBP 4887 and 4888), that did not fit any of the 23 known O-serogroups (Table 1). Two strains, deficient in their LPS sidechains, reacted as RIB (*P. sav. phaseolicola* 4851 and *P. syr. tabaci* 4857). Two new O-serogroups were found within the previously described PHA-MOP cross-reacting group, PHA2 and MOP4 (Table 3). Not all of the strains of *P. sav. phaseolicola* reacted in the same way as the pathovar type strain CFBP 1390 that was typed as PHA1 (O-serogroup previously designated by PHA). Four pv. *phaseolicola* strains were typed as MOP3, the strains isolated from kudzu plants typed as PHA2, and the three strains isolated from *Vigna radiata* typed as APTPIS. Four *P. syr. tabaci* strains isolated from bean were typed as TAB as the type strain of the pathovar (tobacco strain), whereas CFBP 4861 was typed as PHA1. The *P. syringae* species strains CFBP 3650 and 3662 belonged to the serogroup SYR1.

Quantitative DNA hybridisation with P. sav. phaseolicola *pathotype strain*

Quantitative DNA hybridisations were performed between the pathotype strain 1390 of *P. sav. phaseolicola* and 17 strains: 15 *P. sav. phaseolicola* strains belonging to the nine races and the two *P. syringae* strains 3650 and 3662 of unknown pathovar (Table 1). Fifteen strains, independantly of the race or the host plant of origin, showed a reassociation higher than 83%, indicating that they belong to the same genomospecies 2. The two strains 3650 and 3662, giving only 50% and 51% reassociation, did not belong to the genomospecies 2. Therefore, they cannot be designated as *P. savastanoi*.

Comparison of BOX-PCR genomic fingerprinting

The genetic relatedness of the 62 strains of *P. syringae* group, was investigated by BOX-PCR. Following

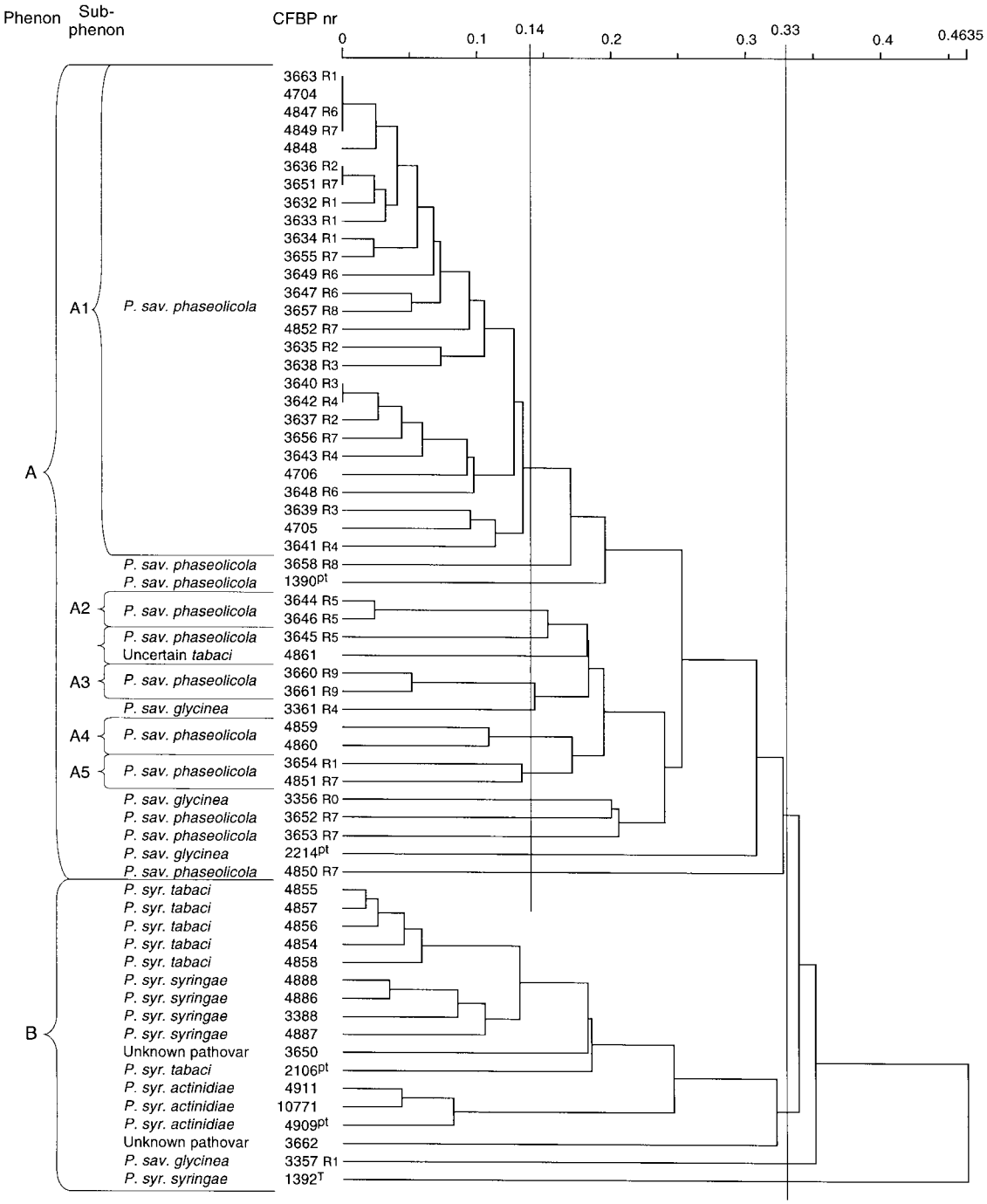

Figure 1. Dendrogram of the distances between 41 *Pseudomonas savastanoi* pv. *phaseolicola* strains and strains of *Pseudomonas syringae* pathovars and related species, tested for 119 phenotypic characteristics. ^{T}Species type strain, ^{Pt}pathotype strain, R = race of *P. sav. phaseolicola* as designated by Taylor et al. (1996) or race of P. *sav. glycinea* as designated by Cross et al. (1966).

Table 2. Assimilation of carbon sources that differentiate phena and isolated strains delineated in Figure 1

Phena (Nr of strains) or isolated strains	Sorbitol	Meso-tartrate	Myo-inositol	D(+)arabitol	D-mannitol	dl-glucuronate	p-OHbenzoate	Trans-aconitate	D(+)malate	L(+)arabinose	Propionate
Phenon A											
A1[a](27)	−	−	−	−	−	+[b]	+	+	+	+	85[c]
A2 (2)	−	−	−	+	+	−	+	+	+	+	−
A3 (2)	−	−	−	+	+	−	+	+	+	+	+
A4 (2)	−	−	−	+	+	+	+	+	+	+	+
A5 (2)	−	−	−	+	+	+	+	−	+	+	+
3658 *P. sav. pha*	−	−	−	−	−	−	+	+	+	+	−
1390[t] *P. sav. pha.*	−	−	−	−	−	−	+	+	+	+	−
3645 *P. sav. pha.*	−	−	−	−	+	−	+	+	+	+	−
4861 *P. syr. tab.*	−	−	+	+	+	−	+	+	+	+	+
3361 *P. sav. gly.*	−	−	+	+	+	+	−	+	+	+	+
3356 *P. sav. gly.*	−	−	−	+	+	−	−	+	+	+	−
3652 *P. sav. pha.*	−	−	−	+	+	+	+	+	−	−	−
3653 *P. sav. pha.*	−	−	−	+	+	−	+	+	+	−	−
2214[t] *P. sav. gly.*	−	−	+	+	+	−	+	+	+	+	−
4850 *P. sav. pha.*	−	−	−	+	+	+	+	−	−	+	+
Phenon B	+	+	+	+	+	+	86	+	+	+	46
3357 *P. sav. gly.*	−	+	+	+	+	+	−	+	+	+	+
1392[T] *P. syr. syr.*	+	−	+	+	+	−	−	−	−	+	+

[a] List of strains in Figure 1.
[b] + or – positive or negative for more than 90% strains.
[c] Percentage of positive strains.

Table 3. Serological reactions (Ouchterlony double diffusion) that define the two new O-serogroups PHA2 and MOP4

Strains	Antisera				O-serogroups[a]
	284	254	286	192	
	(directed against)				
	1390	1650	2351	2115	
1390	+	+	+S	−	PHA1
2115	−	+S	+	+	MOP1
1650	+	+	+	+S	MOP2
2351	+	+	+	+	MOP3
4850	+	−	+	−	PHA2
4886	−	+	+	−	MOP4

[a] According to Saunier et al. (1996).
S: spur between the band of the reacting strain and that of the reference strain.

amplification and agarose gel electrophoresis of resulting PCR products, 12–22 bands were observed for the whole set of strains, and a total of 133 discrete bands were scored, ranging in size from 220 to 3.6 kb. The dendrogram displaying the distances between the 62 strains is shown in Figure 2. At a distance of 0.72, four clusters were delineated; clusters I and III corresponded to genomospecies 2 and 1, respectively (Gardan et al., 1999). The two *P. syringae* strains of unknown pathovars and the three *P. syr. actinidiae* strains were grouped in clusters II and IV, respectively. The great homogeneity of *P. sav. phaseolicola* strains obtained by BOX-PCR fingerprinting is illustrated in Figure 3. Only two polymorphic bands were found, one of the two bands that allows for discrimination is shown on strain 4850. The banding pattern of CFBP 3662 differs from that of the *P. syr. phaseolicola* strains.

REP- and ERIC-PCR fingerprinting, and esterase isozyme profiling of P. sav. phaseolicola *strains*

The patterns generated by the ERIC primers were similar among the 41 *P. sav. phaseolicola* strains, but six distinct profiles could be visualised (Figure 4, Table 4).

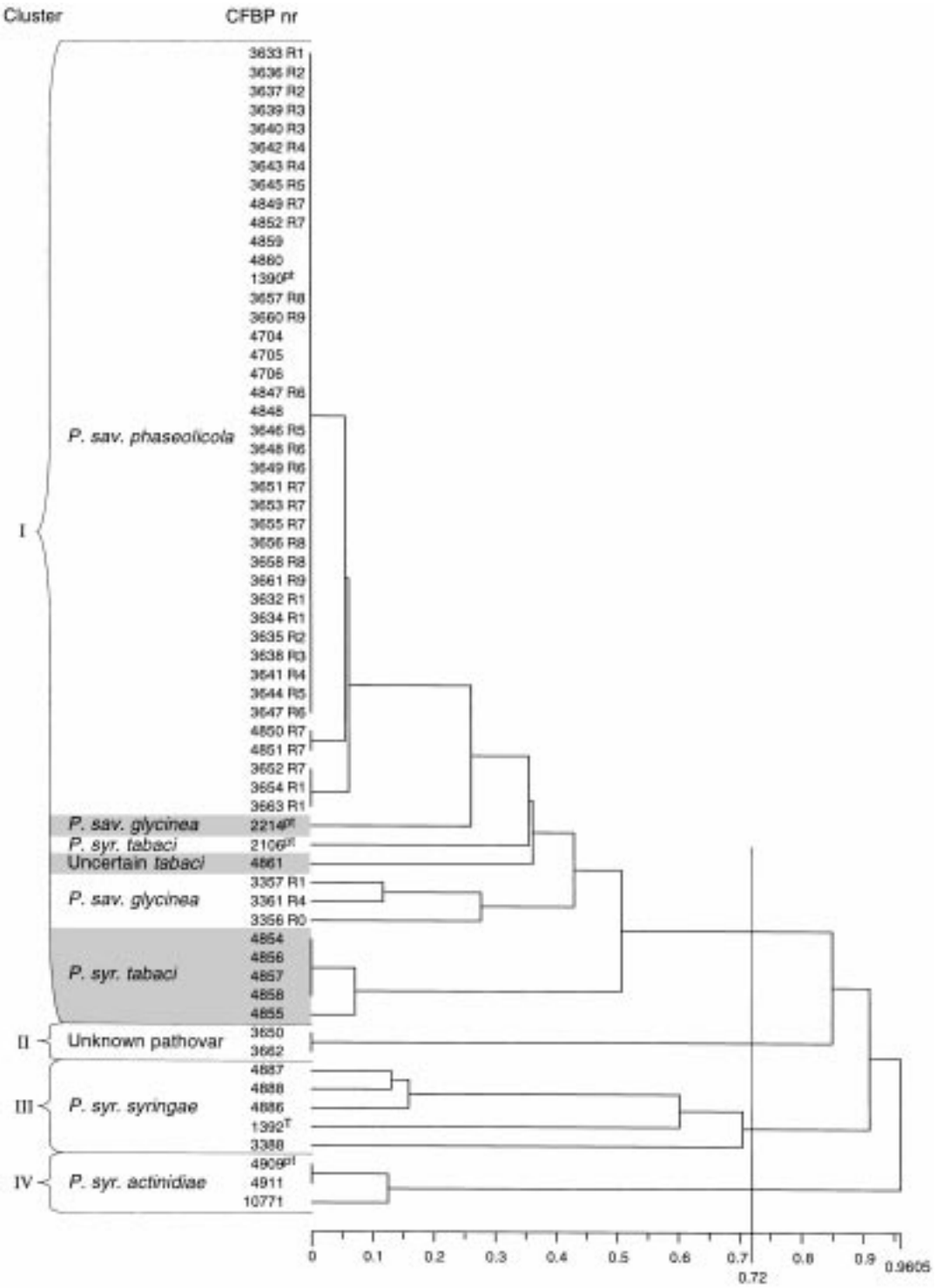

Figure 2. Dendrogram obtained by comparison of BOX-PCR fingerprinting patterns from 41 *Pseudomonas savastanoi* pv. *phaseolicola* strains and strains of *Pseudomonas syringae* pathovars and related species. ^{T}Species type strain, ^{pt}pathotype strain, R = race of *P. sav. phaseolicola* as designated by Taylor et al. (1996) or race of P. *sav. glycinea* as designated by Cross et al. (1966).

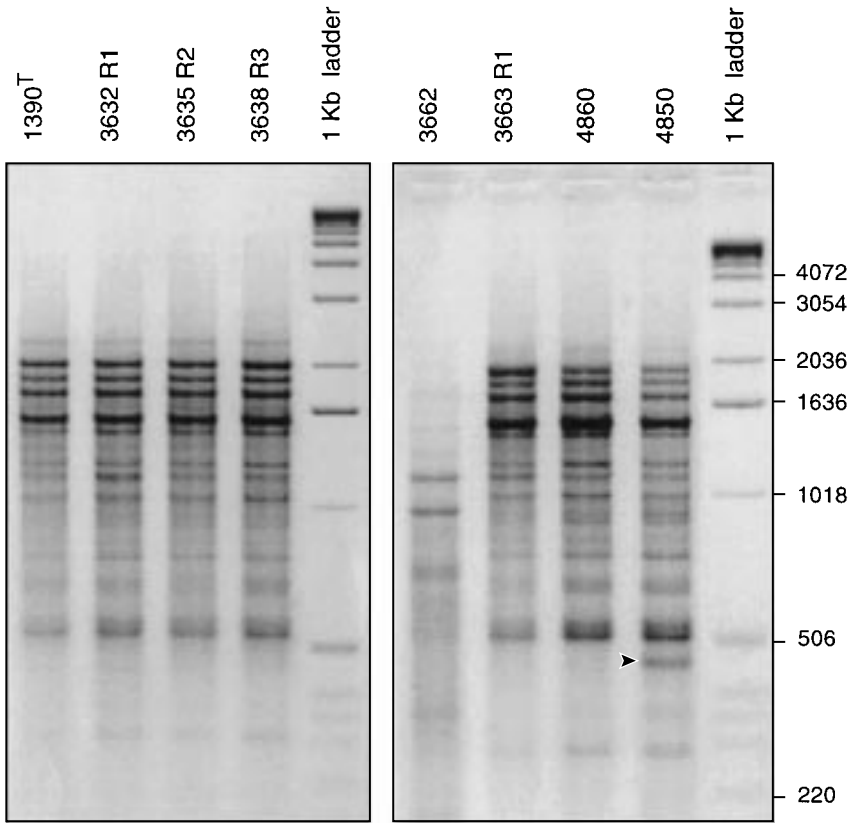

Figure 3. Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of *Pseudomonas savastanoi* pv. *phaseolicola* strains, representing some of the nine races of the pathovar (indicated R1, R2, and R3) and untypable race strains. Arrow indicates rare polylorphism observed among the strains (band BOX-122). Strain 3662 seems quite different. The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Negative image of an ethidium bromide gel shown.

Pattern Nr 1 was assigned to the pathotype strain 1390. The patterns generated by the REP primers were the most diverse obtained in this study and yielded 15 distinct profiles for *P. sav. phaseolicola* (Figure 5, Table 4). Again, pattern Nr 1 was attributed to the pathotype strain. A total of 79 REP- and ERIC-PCR fragments were obtained, ranging in size from ca. 225 bp to 3 kb. Esterase profilings were performed on the 41 strains of *P. sav. phaseolicola* (Figure 6, Table 4) and twelve isozyme bands designated eight zymotypes.

Separate numerical analyses of the three data sets led to similar results, allowing the presentation of all combined data in the dendrogram at Figure 7; four clusters were set up at a distance of 0.3. Cluster A gathered all of the *P. vulgaris* isolates, as well as *P.coccineus*, *Dolichos* sp., *M. atropurpureum*, *L.purpureus*, *N. wightii*, *V. angularis* and *Desmodium* sp. isolates. Cluster B comprised the five strains isolated from kudzu vine *(P. lobata)*, and cluster C the three strains isolated from mungbean *(V. radiata)*. Thus, REP and ERIC analyses and esterase profilings appeared to discriminate some host-origins inside *phaseolicola* pathovar.

RAPD fingerprinting of P. sav. phaseolicola *strains*

RAPD fingerprinting was assayed with two primers (AE7 and AE10) for the 41 *P. sav. phaseolicola* strains. A total of 49 RAPD fragments, from about 249 bp to 2.4 kb, were obtained. From the AE7 primer amplification, three similar profiles could be differentiated by two polymorphic bands (Figure 8, Table 4). From the AE10 primer amplification, five different profiles were

obtained within *P. sav. phaseolicola* differing from each other by three main bands (Figure 9, Table 4).

Cluster analysis of the combined data sets is shown in Figure 10. At a distance of 0.11, the 41 strains of *P. sav. phaseolicola* studied, were separated into three distinct clusters. Cluster RAPD-A included 21 strains, all of the isolates typed as races 1, 5, 7 and 9, one race was untypable, and a single strain (3636) belonged to race 2. Cluster RAPD-B contained the 18 strains of races 2, 3, 4, 6 and 8, and three race untypable strains. Cluster RAPD-C included two race untypable strains out of the five strains isolated from *P. lobata*. Thus, RAPD analysis with the primers AE7 and AE10 could identify two groups of races within the studied collection of *P. sav. phaseolicola* strains.

Table 4 summarises all the typings of *P. sav. phaseolicola* obtained by the panel of techniques used in this study except BOX profiles that were almost identical. The majority of the strains were found to be identical when compared by LPS-serology (29 strains out of 41), by esterase isozyme profiling (29 strains were ZT1), by nutritional criteria (27 strains were A1) and by ERIC-PCR analysis (26 strains for the profile 1). The total number of strains displaying all four characteristics in common was 24. The 18 remaining strains differed either by nutritional criteria, serology, isozyme profile or ERIC-PCR pattern. None of them gave the same response to the four characterisations. When the 24 apparently identical strains of *P. sav. phaseolicola* were analysed by REP-PCR and RAPD, more diversity was observed, and overall, only eight strains displayed the same reactions in all the tests. This group of eight strains originated from diverse geographical areas (Colombia, France, Lesotho, Rwanda, Tanzania and Yemen), two host origins (*P. vulgaris* and *Dolichos* sp.) and harboured strains of races 2, 3, 4, 6, and 8.

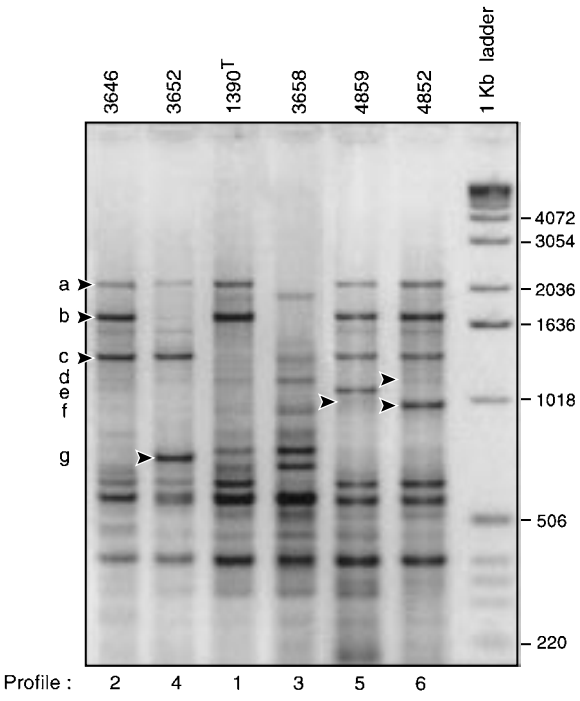

Figure 4. Agarose gel electrophoresis of ERIC-PCR fingerprinting patterns from genomic DNA of *Pseudomonas savastanoi* pv. *phaseolicola* strains, showing the different profiles obtained. Arrows indicate the polymorphic bands among the profiles, a–g for bands ERIC-1, 3, 8, 11, 14, 15 and 20 respectively. The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Negative image of an ethidium bromide gel shown.

Discussion

The assessment of the phenotypic and genetic diversity of a collection of bean pathogenic pseudomonads and reference bacteria showed that *P. sav. phaseolicola*, *P. syr. tabaci* and *P. sav. glycinea* belonging to the genomospecies 2, could be separated at species level from *P. syr. syringae* (genomospecies 1) and from *P. syr. actinidiae* (unknown genomospecies) by BOX-PCR and DNA–DNA hybridisation. To identify *P. sav. phaseolicola* at pathovar level, it was necessary to perform either a pathogenicity test, or nutritional characterisation, serology, together with PCR with specific primers. The diversity revealed within the pathovar *phaseolicola* was linked to its subdivision into pathogenic races when performing RAPD analysis, and linked to the host origin when performing ERIC- and REP-PCR analysis and esterase profiling. The three strains CFBP 3650, 3662, and 4861, either unknown or mislabelled pathovars, were clearly differentiated in all of the analyses.

Diversity of bacterial populations and taxonomy were traditionally studied by phenotypic descriptions but this approach had some limits. A multiphasic approach has been proposed as being a reliable method of integrating different types of information, such as genotypic, phenotypic and phylogenetic data (Colwell, 1970; Vandamme et al., 1996). New methods of fingerprinting based on the analysis of the total genome may constitute a valuable complement (Bruijn, 1992).

Table 4. Summary of the different typings of the 41 *P. sav. phaseolicola* studied

Strain	O-sero group	Zymo type	Nutritional phenon	ERIC profile	REP profile	AE7-RAPD profile	AE10-RAPD profile
3640 3641 3648 3656 4706 3637 3638 3639	PHA1	ZT1	A1	1	4	1	2
3657	PHA1	ZT1	A1	1	9	1	2
4849	PHA1	ZT1	A1	1	2	1	1
3647 4704 4847	PHA1	ZT1	A1	1	8	1	2
3642 3643	PHA1	ZT1	A1	1	5	1	2
4848	PHA1	ZT1	A1	1	3	3	3
3635	PHA1	ZT1	A1	1	3	1	2
3632 3633 3655	PHA1	ZT1	A1	1	1	2	1
3634 3636 4705	PHA1	ZT1	A1	1	2	2	1
3651	PHA1	ZT1	A1	1	10	1	1
3649	PHA1	ZT1	A1	3	3	1	2
3658	PHA1	ZT1	isol. phen.	3	9	1	2
3660	PHA1	ZT1	A3	2	6	2	3
1390^r	PHA1	ZT1	isol. phen.	1	1	1	1
3646	PHA1	ZT7	A2	2	7	2	3
3644	MOP3	ZT1	A2	2	6	2	3
3645	MOP3	ZT6	isol. phen.	2	7	2	3
3661	MOP3	ZT6	A3	2	6	2	3
3653	MOP3	ZT7	isol. phen.	2	6	2	3
3652	APTPIS	ZT5	isol. phen.	4	11	2	1
3654	APTPIS	ZT5	A5	4	12	2	1
3663	APTPIS	ZT5	A1	4	8	1	4
4852	PHA2	ZT2	A1	6	15	2	4
4859 4860	PHA2	ZT3	A4	5	13	2	5
4850	PHA2	ZT4	isol. phen.	5	14	2	3
4851	RIB	ZT3	A5	5	14	2	3

isol. phen.: isolated phenotype.

Ever since the first description of the pathogen *P. sav. phaseolicola*, a pathogenicity test on bean pods was advised (Burkholder, 1926). Compatible and incompatible distinct reactions appeared more clearly on pods than on leaves, because of a prolonged biotrophic phase of infection (Harper et al., 1987). Among the different reactions obtained in this study, lesions induced by *P. sav. phaseolicola* remained specifically watersoaked for 7–9 days. In contrast, the interpretation of the brown and/or sunken lesions obtained with the other inoculated pathovars appeared difficult, and required bean leaf inoculation. After leaf inoculation of beans, the two unknown strains 3650 and 3662 induced numerous lesions, without haloes, similar to that of the compatible strains. The absence of the chlorotic halo for this assay with *P. sav. phaseolicola* strains, could be explained by too high a temperature in the greenhouse (Nuske and Fritsche, 1989). All the *P. syr. tabaci* strains isolated from beans were undoubtedly pathogenic for bean leaves and produced a halo suggesting the tabtoxin effect. The wide halo appeared clearly yellow, and it was not inhibited by the temperature in contrast with the *P. sav. phaseolicola* strains inoculated at the same time (Sinden and Durbin, 1969; Ribeiro et al., 1979).

Nutritional features still constitute an important component of a multiphasic study (Vandamme et al., 1996). In this work, some nutritional characters helped in the identification of *P. sav. phaseolicola*: sorbitol, meso-tartrate, myo-inositol, D(+)arabitol, and D-mannitol. The last three carbon sources allow differentiation from *P. sav. glycinea* and CFBP 4861. This study confirms that the two pathovars *phaseolicola* and *glycinea* are very closely related (Sands et al., 1970). However, additional discriminating characters such as serology can be proposed: O-serogroup PHA for *P. sav. phaseolicola* strains (except *V. radiata* isolates) and APTPIS for *P. sav. glycinea* strains

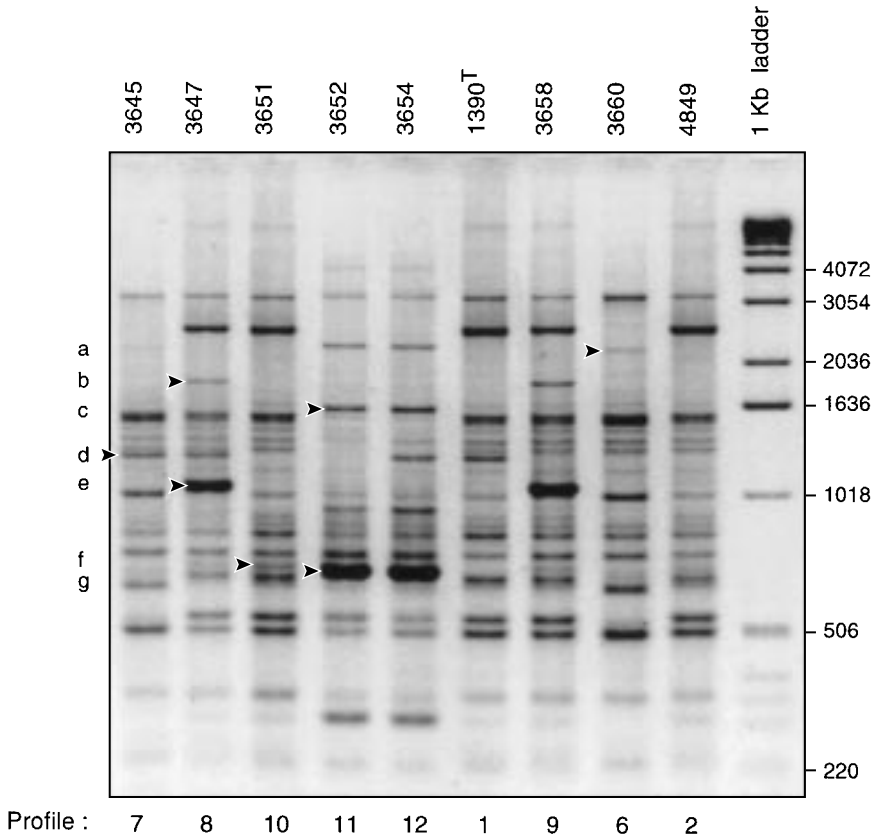

Figure 5. Agarose gel electrophoresis of REP-PCR fingerprinting patterns from genomic DNA of *Pseudomonas savastanoi* pv. *phaseolicola* strains, showing some of the different profiles obtained. Arrows indicate the polymorphic bands among profiles, a–g for bands REP-3, 6, 8, 18, 24, 29, and 36, respectively. The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Negative image of an ethidium bromide gel shown.

(Saunier et al., 1996), and specific gene recognition (Prosen et al., 1993). As well as *P. syr. tabaci* from tobacco, all but one *P. syr. tabaci* strain isolated from beans belonged to the O-serogroup TAB, shown to characterise the tobacco pathogen (Saunier et al., 1996). The exception, CFBP 4861, considered as uncertain pathovar *tabaci*, belonged to the O-serogroup PHA1. *P. syr. tabaci* organisms were isolated several times from diseased soybean in the U.S.A. and in Australia, and two strains kept in the international collections belonged to MOP2 O-serogroup (Saunier et al., 1996). It is generally reported that *P. syr. tabaci* strains isolated from soybean are not pathogenic for tobacco, and vice versa (Clayton, 1950; Ribeiro et al., 1979). In Brazil, *P. syr. tabaci* strains, which were described on bean and pea, showed physiological criteria identical to *P. syr. tabaci* strains isolated from tobacco (Ribeiro et al., 1979) as did five of the strains in our study. A diversity study of *P. syr. tabaci* populations on their different host plants in the countries where they provoke losses, would be interesting with the new techniques now available.

Phaseolotoxin (Mitchell, 1976) was shown to be produced by strains belonging to other pathovars than *P. sav. phaseolicola*, as was also the case for *P. syr. actinidiae* (Tamura et al., 1989) and the strain CFBP 3388 of *P. syr*. *syringae* isolated from *Vicia sativa* (Tourte and Manceau, 1995). We want to point out that not all of the *P. syr. actinidiae* strains tested reacted positively to our PCR test. This heterogeneity has not been reported by Sawada et al. (1997). Concerning the PCR specificity, *P. syr. actinidiae* strains, pathogenic to kiwifruit, are not likely to be found on bean plants or seed, but a detection test based

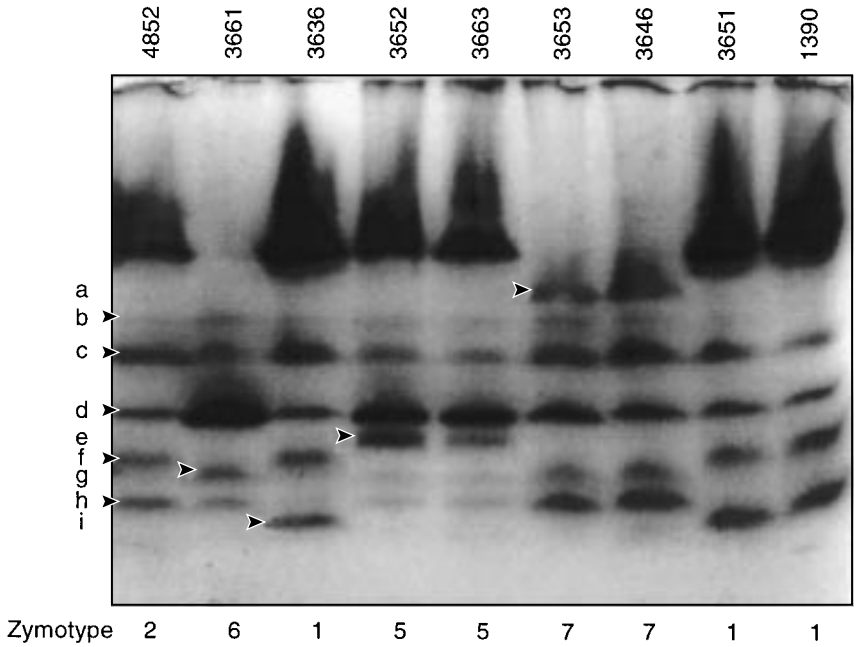

Figure 6. PAGE of esterase isozymes from *Pseudomonas savastanoi* pv. *phaseolicola* strains, showing the different zymotypes obtained. Arrows indicate the various isomorphs (a–i).

only on PCR recognition would be confused by the existence of strains of *P. syr. syringae* similar to CFBP 3388 in the epiphytic flora. One can predict that if such strains occur, it would be unlikely for them to reach a population level high enough to be detected in seed health testing assays.

Serological characterisation constitutes a helpful technique especially when the O-serogroup of the studied pathogen is not frequent among the *P. syringae* organisms (Grondeau et al., 1992; Samson et al., 1998). The O-serogroup PHA was only reported for *P. sav. phaseolicola* (Saunier et al., 1996) in a study dealing with 15 pathovars of the *P. syringae* group. It was demonstrated to be exclusively recognised by a monoclonal antibody (Ps1c) by Ovod et al. (1999). However, the O1c specific epitopes are also borne by *P. syr. actinidiae* (this study), and *P. syr. atrofaciens*, *P. syr. avellanae*, and *P. syr. tagetis* (Ovod et al., 1999). Conversely, three *P. sav. phaseolicola* strains (*V. radiata* isolates) belong to a second O-serogroup, APTPIS (corresponding to the monoclonal antibody Ps1d, Ovod et al., 1999), the same serogroup that characterises *P. syr. aptata*, *P. syr. pisi* and *P. sav. glycinea*. This rare O-serogroup for *P. sav. phaseolicola* constitutes a good indicator that *V. radiata* isolates are clearly different from the other strains of the pathovar.

Flagella serotyping showed that all *P. sav. phaseolicola*, *P. sav. glycinea*, *P. syr. tabaci*, and *P. syr. actinidiae* strains tested bore the H2 serotype. Only *P. syr. syringae* strains presented H1 serotype. Our results are consistant with a previous study where it was shown that flagella remained as highly conserved antigens within the *P. syringae* group (Malandrin and Samson, 1999). H1 flagella are borne by pathovars belonging to the genomospecies 1, 4, 7, and 9, whereas H2 flagella are borne by strains of the genomospecies 2, 3, 6, and 8, as defined by Gardan et al. (1999). Therefore, CFBP 3650 and 3662 cannot be regarded as belonging *to P. syringae* genomospecies 1.

Extragenic repetitive sequences were first documented by Versalovic et al. (1991) and Martin et al. (1992) to directly fingerprint bacterial genomes. In this study, we examined a total of 62 strains of the *P. syringae* group, comprising 51 bean pathogens, by the fore-mentioned rep-PCR techniques. When using BOXA1R primer to fingerprint the genome of all of these strains, the results showed a great homogeneity for the pathovar *phaseolicola*, and few differences inside the other members of the genomospecies 2

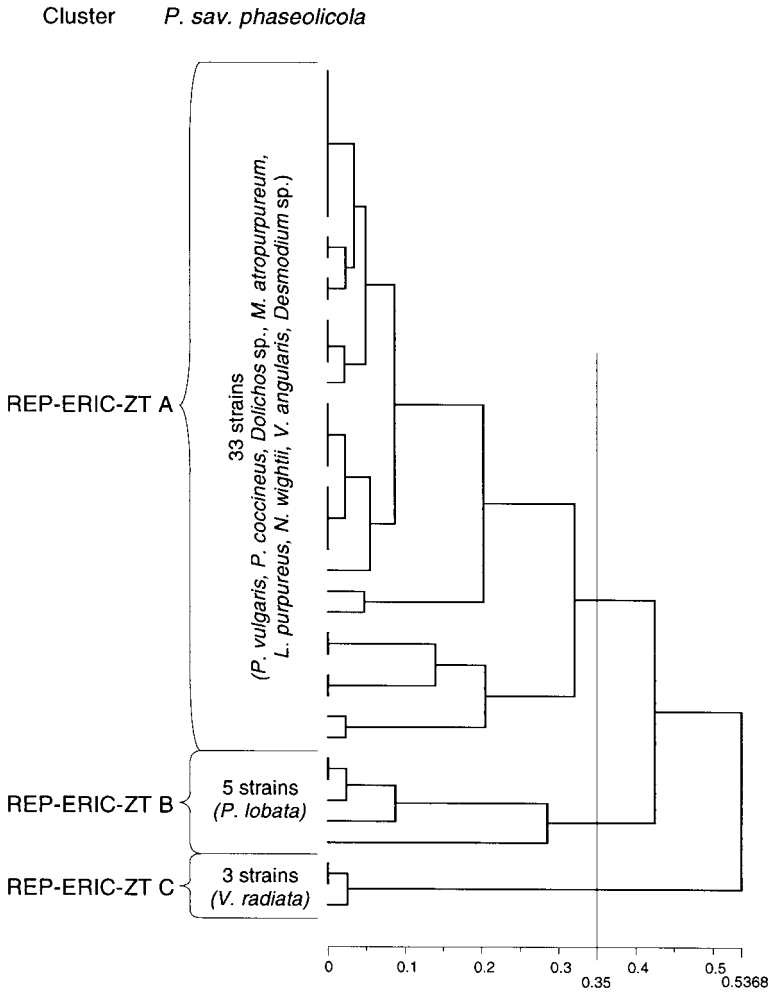

Figure 7. Dendrogram obtained by comparison of REP-/ERIC-PCR and esterase isozyme fingerprinting patterns from 41 *Pseudomonas savastanoi* pv. *phaseolicola* strains representing the nine races of the bacterium (as designed by Taylor et al., 1996) as well as the pathovar type strain and untypable race strains.

(*P. savastanoi*), as described by Gardan et al. (1999). The same relative homogeneity was observed inside the genomospecies 1, and the numerical analysis showed that BOX primer may be sufficiently powerful to discriminate bacteria at genomospecies level, as well as *Sma*I and *Hinc*II ribotyping patterns and DNA/DNA hybridisation (Gardan et al., 1999). A work performed on the *Xanthomonas* group already indicated that the rep-PCR generated groupings that correspond directly to the DNA homology groups (Bruijn, 1996). The present paper is the first report that the BOX-PCR results could be correlated with species discrimination, discrimination based on quantitative DNA/DNA hybridisation as recommended by Wayne et al. (1987). A thorough study of all the pathovars of *P. syringae* group is under way to check this hypothesis.

BOX-PCR revealed the homogeneity of *P. sav. phaseolicola*. RAPD, REP- and ERIC-PCR fingerprinting were useful for studying the genetic diversity of this pathovar, because of the intrapathovar variation. When all three rep-PCR techniques were shown to be suitable for the characterisation of the pathovars of *P. syringae* (Louws et al., 1994), it was not yet established that the three pathovars studied (*morsprunorum*, *syringae*, and *tomato*) belonged to three different genomospecies 2, 1, and 3, respectively.

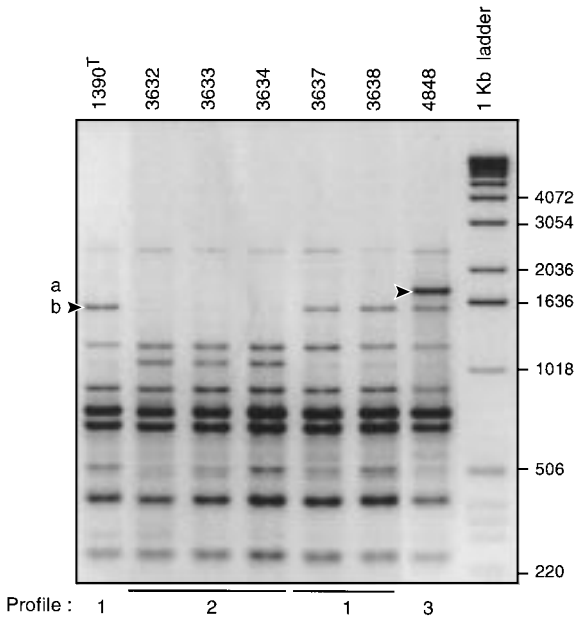

Figure 8. Agarose gel electrophoresis of of RAPD fingerprinting patterns from genomic DNA of *Pseudomonas savastanoi* pv. *phaseolicola* strains, obtained from primer AE7 showing the different profiles obtained. Arrows indicate the polymorphic bands AE7-2 and AE7-4 among the profiles 1, 2 and 3. The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. The negative image of an ethidium bromide gel is shown.

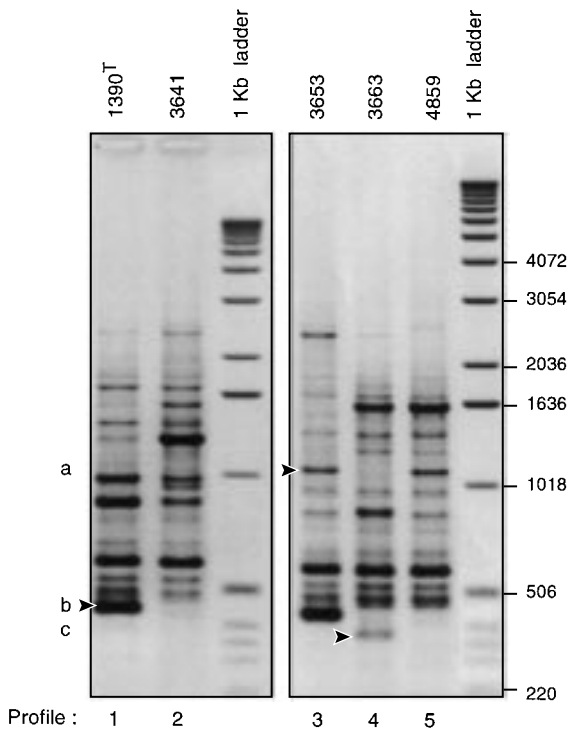

Figure 9. Agarose gel electrophoresis of RAPD fingerprinting patterns from genomic DNA of *Pseudomonas savastanoi* pv. *phaseolicola* strains, obtained from primer AE10 showing the different profiles obtained. Arrows indicate the polymorphic bands among the five profiles, a–c for bands AE10-13, AE10-29 and AE10-30 respectively. The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. The negative image of an ethidium bromide gel is shown.

REP primers were reported to yield, for *E. coli*, a great variety of patterns that differed from each other by very few bands (Versalovic et al., 1991). REP and ERIC analyses enable us to group the strains according to their plant host, at least in two cases: the strains isolated from kudzu (*P. lobata*) and those from mungbean (*V. radiata*). Weingart and Völksch (1997) already separated strains of *P. sav. phaseolicola* from bean and kudzu plants by ERIC primers. They suggested the kudzu strains could constitute a separate pathovar, distinct from pv. *phaseolicola*. Actually, the kudzu strains display a particular set of characters inside the pv. *phaseolicola*: REP and ERIC patterns, esterase zymotypes, O-serogroup (this study), and an overlapping host range that included soybean, kudzu vine and common bean (Völksch and Weingart, 1997). The question needs to be debated. By means of RAPD primers, strains of *P. syr. tomato*, *P. syr. apii* and *P. syr. maculicola* (now belonging to the genomospecies 3) were discriminated at the pathovar level (Little and Gilbertson, 1997; Clerc et al., 1998).

Birch et al. (1997) also obtained by RAPD a very clear distinction between *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*, that allowed them to design specific primers for the detection by standard PCR of the two variants. RAPD analysis did not group the 41 *P. sav. phaseolicola* strains the same way as REP and ERIC-PCR. RAPD primers revealed two groups of strains, allocating them to the same race affiliation (races 1, 5, 7, and 9, and races 2, 3, 4, 6, and 8) as described by Taylor et al. (1996). González et al. (1998) designed scars from RAPD polymorphic bands, allowing them to separate strains classified as races 1 and 2 by standard PCR. However, it is not clear if they could follow the race classification up to nine races. The two groups of races would indicate two evolutionary lines, diverging according to their pathogenicity for the bean cultivar 'Red Mexican'.

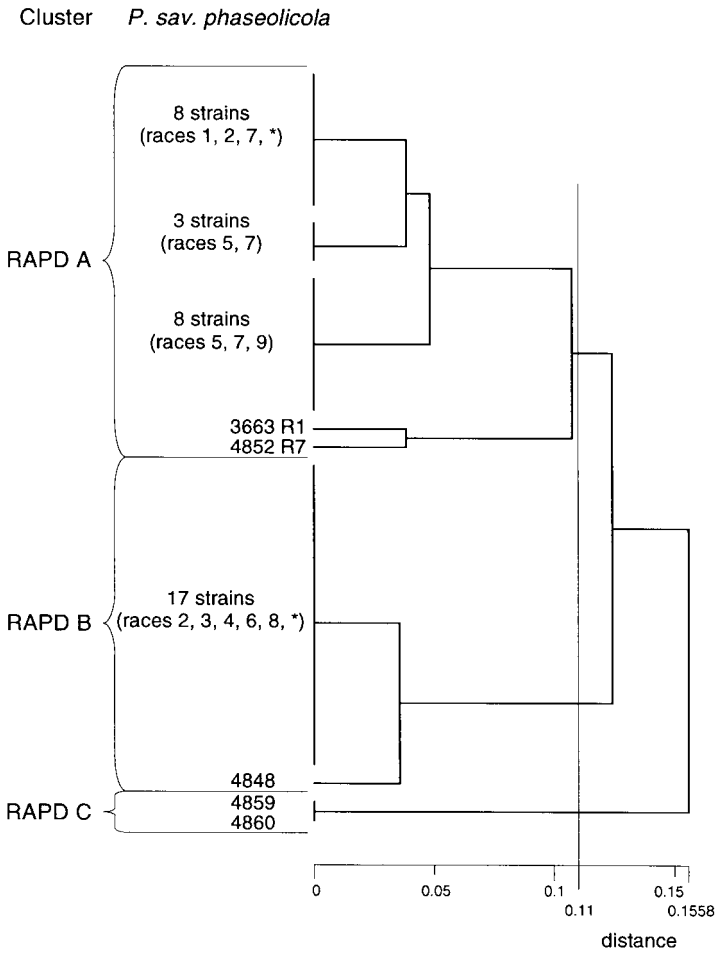

Figure 10. Dendrogram obtained by comparison of RAPD fingerprinting patterns from 41 *Pseudomonas savastanoi* pv. *phaseolicola* strains representing the nine races of the bacteria (as designed by Taylor et al., 1996) as well as the pathovar type strain and unknown race type strains. ∗ including untypable race strains.

The group of strains compatible with 'Red Mexican' seems much more homogeneous in our study, than the group comprising races 1, 5, 7, and 9 that are incompatible. The strains of the latter group must possess many more genetical differences than the corresponding *avr* genes.

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