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Specific oligonucleotide primers for the rapid identification and detection of the agent of tomato pith necrosis, *Pseudomonas corrugata*, by PCR amplification: evidence for two distinct genomic groups

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Abstract

Unique DNA bands from strains representative of two groups of *Pseudomonas corrugata*, as shown by amplification of their genomic DNA by polymerase chain reaction using short random sequence oligonucleotide primers (RAPD-PCR), were isolated, cloned and sequenced. Two pairs of specific primer sequences, based on the ends of the cloned unique DNA bands from strains IPVCT10.3 and IPVCT8.1, were used in multiplex PCR with a range of *P. corrugata* strains. All strains produced one of the two specific bands, 1100 bp (from the IPVCT10.3-based primers) and 600 bp (from the IPVCT8.1-based primers), representing groups designated I and II, respectively. The primers were also tested on a wider range of *Pseudomonas* species, including the closely-related fluorescent *Pseudomonas* genomospecies FP1, FP2 and FP3: none of these bacteria produced any bands following amplification by PCR with these primers. The primer sets detected *P. corrugata* in tomato pith necrosis-infected plants providing a useful tool for rapid identification and epidemiological studies.

Introduction

Pseudomonas corrugata Roberts & Scarlett is the causal agent of tomato pith necrosis (TPN). The disease occurs world-wide in all tomato-growing areas and while resembling an opportunistic bacterium, also causes severe crop losses (Scarlett et al., 1978; Bradbury, 1987; Catara and Albanese, 1993; Lopez et al., 1994). Characteristic symptoms of the disease are the necrosis and/or hollowing of the parenchymatic tissue of the stem. Frequently, the first visible symptom is chlorosis of the youngest leaves. This often happens on plants where the fruit of the first truss is fully grown (Scarlett et al., 1978). With increasing extension of pith necrosis, the plant loses turgor and collapses. Significant losses occur on tomatoes grown in unheated glasshouses, where large diurnal temperature extremes and high humidity seem to favour the development of the disease (Scarlett et al., 1978). *P. corrugata* has also been isolated from pepper (Lopez et al., 1988), chrysanthemum (Fiori, 1992) and geranium (Magyarosy and Buchanan, 1995) associated with induced pith necrosis.

The bacterium is a non-fluorescent *Pseudomonas* species that accumulates poly-β-hydroxybutyrate. Nevertheless, both on the basis of DNA–rRNA hybridisation data (De Vos et al., 1985) and on fatty acid profiles (Stead, 1992), *P. corrugata* belongs to the fluorescent pseudomonads. The emended description of the species points out, on the basis of phenotypic analysis and DNA–DNA reassociation results, that *P. corrugata* is very closely related to fluorescent *Pseudomonas* strains associated with tomato pith necrosis (FPTPN), that have been proposed as three un-named *Pseudomonas* genomospecies (FP1, FP2 and FP3), and more related to fluorescent *Pseudomonas* species

than to non-fluorescent *Pseudomonas*, *Acidovorax*, *Herbaspirillum* and *Ralstonia* species (Sutra et al., 1997).

Traditional methods for the detection and identification of *P. corrugata* rely on isolation of the organism in pure culture and performance of specific biochemical, serological and pathological tests (Siverio et al., 1993; Sutra et al., 1997). Attempts to find rapid methods for the identification of *P. corrugata*, have encountered problems associated with the high phenotypic and genomic variability observed in the species (Siverio et al., 1993; 1996; Catara et al., 1997; Sutra et al., 1997).

PCR-based detection and identification protocols have been described for diverse groups of plant-pathogens (Henson and French, 1993). The random amplified polymorphic DNA (RAPD) PCR method, used with a number of bacterial species to fingerprint genomes, enabled identification of genomic targets for specific PCR assays (Manulis et al., 1994; Arnold et al., 1996; Pooler et al., 1996). The aim of this study was to use RAPD-PCR-generated fragments to design oligonucletotide primers, capable of specific and sensitive use in multiplex PCR for the identification of *P. corrugata* and which would be potentially useful for direct detection of the bacteria in infected tissues.

Materials and methods

Bacterial strains and plasmids

The strains of *P. corrugata* examined, which included 30 from the culture collection of the Istituto di Patologia Vegetale, Università degli Studi di Catania, Italy (IPVCT), were from collections world-wide (Table 1). The pathogenicity and taxonomy of these strains were verified by standard procedures (Catara et al., 1997). A number of pseudomonad strains belonging to other species were also tested (Table 1). All *P. corrugata* strains were maintained on nutrient agar or broth (DIFCO, Detroit, MI, USA) supplemented with 1% D-glucose (NDA) and all the other pseudomonads on King's medium B (King et al., 1954). *Escherichia coli* strains were grown overnight in Luria broth (LB) (Sambrook et al., 1989) or on LB agar at 37 °C. All bacteria were stored long term at −80 °C in LB with 15% glycerol. Plasmids derived from pCR2.1 (Invitrogen, Groningen, the Netherlands) were pAV619, containing a 650 bp RAPD fragment obtained with primer OPA9 (Operon Technologies, USA) from *P. corrugata* strain IPVCT8.1 and pAV620, containing a 1100 bp RAPD fragment obtained with primer OPA10 from *P. corrugata* strain IPVCT10.3. These were cloned using the original TA cloning kit (Invitrogen, Groningen, the Netherlands) in *E. coli* strain INVαF' (Invitrogen, Groningen, the Netherlands), as described by the manufacturer.

DNA extraction, purification, cloning and sequencing

Total genomic DNA was extracted from 1.5 ml broth cultures with the Puregene DNA isolation kit (Gentra System Inc., Flowgene, Lichfield, UK). The quantity of DNA was estimated by comparison with known standards in ethidium bromide-stained 1% agarose gels. RAPD bands of interest were purified from agarose gels with QIAEX II gel extraction kit (QIAGEN, Dorking, UK). Plasmid DNA was isolated by alkaline lysis (Sambrook et al., 1989) for dot-blot hybridisation analysis and with a QIAGEN Plasmid Midi kit (QIAGEN, Dorking, UK) for sequence analysis. Plasmid inserts were partially sequenced using an Amplicycle Sequencing kit (Perkin Elmer, Warrington, UK) in a Macrophor sequencing apparatus (Pharmacia, St. Albans, UK). Oligonucleotides designed from the sequences were synthesised by Genenco (M-Medical, Italy). Restriction enzymes (Gibco BRL, Life Technologies, Italy) were used according to the manufacturer's instructions.

PCR amplification and analysis of PCR products

The PCR reactions were carried out in a 25 μl reaction mix, containing: PCR buffer (HT Biotechnology, Cambridge, UK; Gibco BRL, Life Technologies, Italy), 1.5 mM MgCl2, 20 mM of each dNTP (Gibco BRL, Life Technologies, Italy), the required concentration of each primer and 1.25 U Taq polymerase (HT Biotechnology, Cambridge, UK; Gibco BRL, Life Technologies, Italy). Genomic DNA (20 ng) or bacterial cells from a plate culture were used as the template source. RAPD-PCR was performed using the above reaction mix with 10 pmol of the single OPA primer. The amplification was carried out in a thermal cycler (480 Perkin Elmer, Warrington, UK) programmed for 40 cycles of 30 s at 94 °C, 1 min at 35 °C, 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. Specific amplification of *P. corrugata* DNA was performed with 10 pmol

Table 1. Bacterial strains used in this study, their geographic origin, host of isolation and reaction in PCR with the primers specific to *Pseudomonas corrugata*

Species[1]	Strain	Source[2]	Origin	Host	PCR band[3]
P. corrugata	2445[T]	NCPPB	UK	*Lycopersicon esculentum*	I
	10.3,2.1, 2.2, 3.1, 4.1, 4.2, 7.2,7.6, 7.8, 10.2, 10.6, 10.8, 10.12, 10.14, A1, D1	IPVCT	Italy	*L. esculentum*	I
	4.3.t, 4..5.t, 5.1.t, 5.2.t, 6.1.t	IPVCT	Italy	(Soil)	I
	B002	M. Scortichini	Italy	*L. esculentum*	I
	98a	M. Fiori	Italy	*Chrysanthemum morifolium*	I
	60b	M. Fiori	Italy	*L. esculentum*	I
	8895	ICMP	New Zealand	*L. esculentum*	I
	10058	CFBP	New Zealand	*L. esculentum*	I
	536.7.1, 5.4, 614.5.3, 632.2, 632.5, 1113.5	IVIA	Spain	*L. esculentum*	I
	10900	CFBP	Spain	*L. esculentum*	I
	J375	S. Kohn	Germany	*L. esculentum*	I
	86.16.9	L. Gardan	France	*L. esculentum*	I
	10532	CFBP	France	*L. esculentum*	I
	3056	NCPPB	USA	*Medicago sativa*	I
	3316	NCPPB	South Africa	*L. esculentum*	I
	8.1, 8.2, 8.3, 9.1,3B, 3C, 1.1, 1.4, 1.11, P1	IPVCT	Italy	*L. esculentum*	II
	B021	M. Scortichini	Italy	*L. esculentum*	II
	1.1.6	IVIA	Spain	*L. esculentum*	II
	592.4.4[4]	IVIA	Spain	*Capsicum annuum*	II
	82.23.6	L. Gardan	France	*L. esculentum*	II
	10961, 1894	CFBP	Spain	*L. esculentum*	II
P. agarici	2472	NCPPB	UK	*Agaricus bisporus*	—
P. aeruginosa	2466[T]	CFBP			—
P. cichorii	907	NCPPB	USA	*Chrysanthemum morifolium*	—
P. fluorescens	2102[T]	CFBP	UK	(Water)	—
P. marginalis pv. *marginalis*	667[T]	NCPPB	USA	*Cichorium intybus*	—
P. putida mt-2	PaW340	P.A. Williams	UK	(Soil)	—
P. tolaasii	2192[T]	NCPPB	UK	*Agaricus bisporus*	—
P.glycinea pv. *savastanoi*	3334	NCPPB	France	*Nerium oleander*	—
P. syringae (pv. *syringae*-like)	1480A	HRI	UK	*Pisum sativum*	—
P. syringae pv. tomato	28.2	IPVCT	Italy	*L. esculentum*	—
P. viridiflava	SG17-5	R. Samson	France	*Pisum sativum*	—
FPTPN genomospecies FP1	10435	CFBP		*L. esculentum*	—
FPTPN genomospecies FP2	10529, 10530	CFBP		*L. esculentum*	—
FPTPN genomospecies FP3	10569	CFBP		*L. esculentum*	—
FPTPN	60	IPVCT	Italy	*L. esculentum*	—

[1]FPTPN: fluorescent pseudomonads associated with tomato pith necrosis.
[2]NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; IPVCT: Istituto di Patologia Vegetale, Università degli Studi di Catania, Italy; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; CFBP: Collection Francaise de Bactéries Phytopathogènes, Angers, France; IVIA: Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain; HRI: Horticulture Research International, Wellesbourne, UK; M. Scortichini, Istituto Sperimentale per la Frutticoltura, Roma, Italy; M. Fiori, Istituto di Patologia Vegetale, Università degli Studi di Sassari, Italy; S. Kohn, obtained from IVIA; L. Gardan and R. Samson, INRA, Station de Pathologie Vegetale, Beaucouze, France; P.A. Williams, School of Biological Sciences, University of Wales Bangor, UK.
[3]Band I is 1100 bp; band II is 600 bp; – no amplification products. PCR conditions as described in Materials and methods.
[4]The same strain also tested as CFBP 10148.
[T]Type strain.

of each primer through one cycle of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 62 °C and 1 min 30 s at 72 °C, followed by one cycle of 5 min at 72 °C. Amplified products were resolved by electrophoresis at 5.7 V cm^{-1} in 1.5% agarose in 0.5 × Tris–borate–EDTA-buffer (Sambrook et al., 1989), stained with ethidium bromide and photographed under UV illumination.

Dot-blot analysis of DNAs and hybridisation

Bacterial DNA samples were transferred by vacuum-blotting to a Biotrans (+) nylon membrane (ICN, Basingstoke, UK) as described by Arnold et al. (1996). PCR fragments (extracted from agarose gels) and plasmid inserts were labelled with deoxycytidine 5′ [α-^{32}P] triphosphate using the random primer oligo-labelling method with a High Prime kit (Boehringer Mannheim, Lewes, UK). The blots were hybridised and washed under highly stringent conditions as previously described (Arnold et al., 1996).

PCR detection of P. corrugata *in infected tomato tissues*

For direct detection of *P. corrugata* in tomato tissues, artificially inoculated plants were used. One month-old tomato plantlets of cultivar Arletta were inoculated in the laboratory with a bacterial suspension (50 µl containing 10^8 cfu ml^{-1}) in sterile distilled water (SDW), injected at the axil of the first true leaf. Control plants were inoculated with SDW. Plants were enclosed in clear plastic bags and maintained in a growth chamber (7 days) with 16/8 h day/night photoperiod at 25 °C. From each plant, transverse sections (3 mm thick) of stems showing pith necrosis were transferred singularly to 1.5 ml sterile Eppendorf tubes and processed. The bacterial cells were left to diffuse from the tissue or ground in 200 µl of SDW and 5 µl were added to 495 µl of SDW: both the undiluted and diluted preparations were utilised in PCR. Alternatively, a quick method of DNA extraction was utilised (Audy et al., 1994) and the tissue was left to diffuse or was ground in 200 µl of NaOH 0.5 N with and without polyvinyl pyrrolidone (PVP) and 5 µl transferred in 495 µl of Tris–HCl 20 mM pH 8. Samples (1 and 5 µl) were tested by PCR amplification. Viable counts from the samples were determined on NDA.

Results

RAPD-PCR

Representative isolates of *P. corrugata*, chosen on the basis of their different phenotypic characteristics (Catara et al., 1997), were subjected to RAPD-PCR with nine different primers of which five (OPA1, OPA4, OPA9, OPA10 and OPA20) clearly separated the strains into two groups. Group I contained the type strain NCPPB2445 and strains NCPPB3056, IPVCTA1 and IPVCT10.3, and group II contained strains IPVCT8.1 and IPVCT9.1 (Figure 1). Amplified fragments were isolated from the gels, radiolabelled with ^{32}P-dCTP and used as probes against dot-blots of the representative *P. corrugata* isolates (data not shown). Two fragments were identified. One fragment from isolate IPVCT8.1 generated with OPA 9, strongly hybridised with strains IPVCT8.1 and IPVCT9.1 and slightly

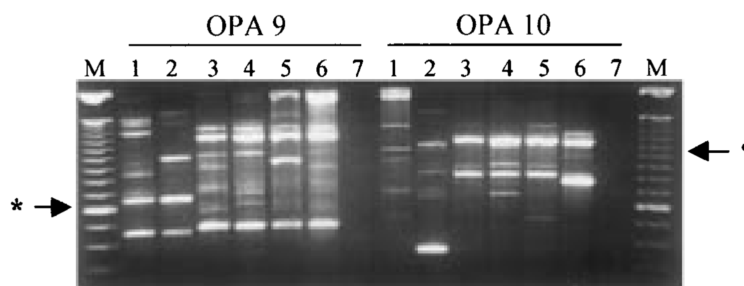

Figure 1. RAPD-PCR analysis of representative *P. corrugata* strains with two 10-mer oligonucleotides (OPA 9 and OPA 10). Lanes 1, IPVCT9.1; 2, IPVCT8.1; 3, IPVCT10.3; 4, IPVCTA1; 5, NCPPB2445, type strain; 6, NCPPB3056; 7, no DNA control; M, 100 bp ladder. Arrows indicate bands selected for further work: * 650 bp, from strain IPVCT8.1 with OPA9; • 1100 bp, from strain IPVCT10.3 with OPA 10.

with the other strains. The other fragment, from strain IPVCT10.3, generated with OPA 10, hybridised only to isolates IPVCT10.3, IPVCTA1, NCPPB2445 and NCPPB3056.

Cloning and sequencing of specific fragments

The two fragments were cloned in *E. coli* INVαF' as plasmids pAV619 and pAV620 from strain IPVCT8.1 (about 650 bp) and strain IPVCT10.3 (about 1,100 bp), respectively. The insert of plasmid pAV619 was released from the vector by digestion with *Eco*RI; the insert of plasmid pAV620 was released after digestion with *Xba*I and *Bam*HI, because the insert contained an *Eco*RI site. The inserts were used to probe dot-blots of representative isolates of *P. corrugata* and of other pseudomonads. The results (Figure 2) confirmed the pattern of hybridisations performed with the RAPD-PCR DNA fragments and neither fragment hybridised with the other pseudomonads used in the dot-blots (*P. putida* PaW340, *P. marginalis* pv. *marginalis* NCPPB667, *P. tolaasii* NCPPB2192, *P. cichorii* NCPPB907, *P. glycinea* pv. *savastanoi* NCPPB3334, *P. agarici* NCPPB2472, *P. syringae* pv. *syringae* HRI1480A, *P. viridiflava* SG175). The fragments were sequenced in both directions and the resulting sequences were compared to the EMBL/GenBank Nucleotide Sequence Database using

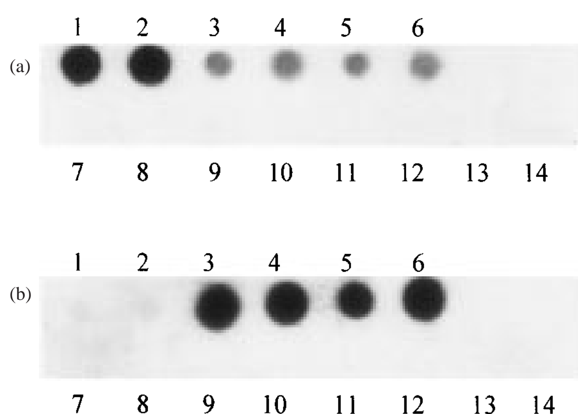

Figure 2. Dot blots of a range of *Pseudomonas* strains probed with the insert clones pAV619 (a) and pAV620 (b). *P. corrugata* strains (upper row): 1, IPVCT9.1; 2, IPVCT8.1; 3, IPVCTA.1; 4, NCPPB2445 type strain; 5, IPVCT10.3; 6, NCPPB3056; (lower row): 7, *P. putida*; 8, *P. marginalis* pv. *marginalis*; 9, *P. tolaasii*; 10, *P. cichorii*; 11, *P. savastanoi*; 12, *P. agarici*; 13, *P. syringae* pv. *syringae*; 14, *P. viridiflava*.

the GCG program FastA but no significant matches were found. Four oligonucleotides primers were designed from the ends of the two sequences: PC1/1 (5′ GGATATGAGCCAGGTCTTCG 3′) and PC1/2 (5′ CGCTCAAGCGCGACTTCAG 3′), the forward and the reverse sequences derived from pAV619, and PC5/1 (5′ CCACAGGACAACATGTCCAC 3′) and PC5/2 (5′ CAGGCGCTTTCTGGAACATG 3′), the forward and the reverse sequences from pAV620.

Development of PCR conditions

By using the conditions described in Materials and methods (10 pmol of each primer and an annealing temperature of 62 °C), amplification products of the expected sizes (600 and 1100 bp) were obtained both with DNA and bacterial cells of representative strains as well as from the plasmids pAV619 and pAV620, respectively. The primer pairs were subsequently designated type I (PC5/1 and PC5/2) and type II (PC1/1 and PC1/2) and the groups of strains were thereby identified as I and II, respectively (Figure 3).

Specificity and sensitivity of the PCR

Since the initial strains examined showed an apparent separation into two distinct groups, it was important to examine a wider range of strains well studied for their phenotypic characteristics. Using all four primers in the reaction mix, DNA from a collection of *P. corrugata* strains and a range of strains representing other *Pseudomonas* spp. was subjected to PCR amplification (Table 1). Specific bands were obtained only with *P. corrugata* strains, which showed one or the other band, and were thus assigned to one of the two designated groups, I and II (Figure 4; Table 1).

The level of detection with PCR was investigated by amplification both from bacterial cells and from genomic DNA. Cell suspensions in SDW of the strains IPVCT10.3 and IPVCT9.1 were serially diluted to 10^{-7} and 1 μl aliquots used in PCR amplification with type I and type II primers, respectively. A band was visible on the gel corresponding to 2×10^3 cfu/PCR reaction with strain IPVCT10.3 and 4×10^3 cfu/PCR reaction with strain IPVCT9.1 (Figure 5). One microliter aliquots of ten-fold dilutions of total DNA of the strains IPVCT10.3 and IPVCT9.1 in the range 20 ng to 20 fg were used for amplification. The detection level for both primer sets was about 20 pg of DNA (Figure 6).

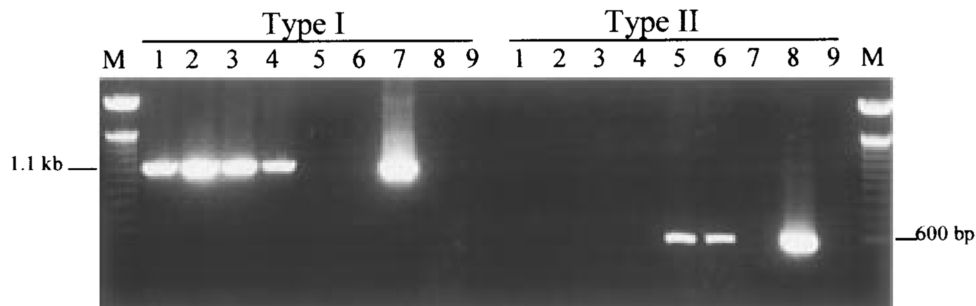

Figure 3. PCR amplification with type I and type II primers of DNA from representative strains of *P. corrugata* and of plasmids containing corresponding cloned inserts. Lanes: 1, IPVCT10.3; 2, IPVCTA.1; 3, NCPPB2445 type strain; 4, NCPPB3056; 5, IPVCT9.1; 6, IPVCT8.1; 7, plasmid pAV620; 8, pAV619; 9, control – no DNA; M, 100 bp DNA ladder.

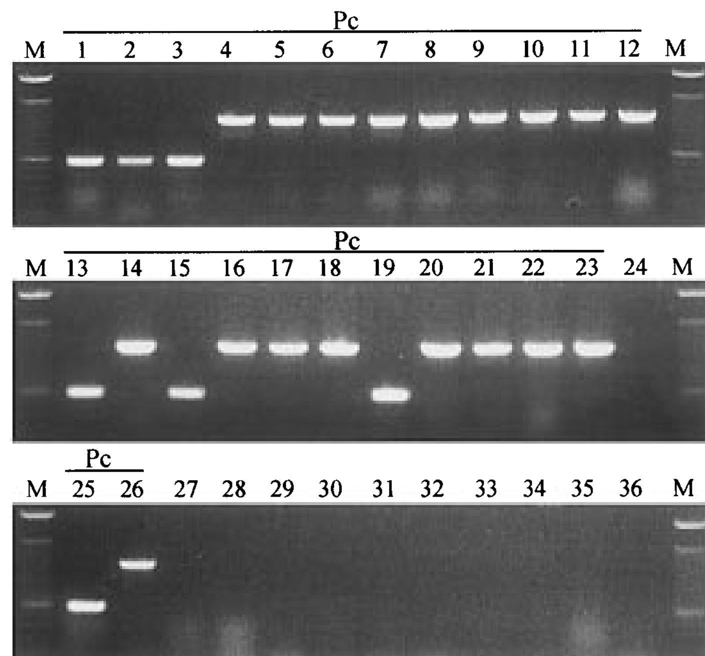

Figure 4. PCR amplification with a mixture of type I and type II primers. Amplification products from various strains of *P. corrugata* (lanes 1–23, 25, 26) and other *Pseudomonas* spp. (lanes 27–36) were separated by agarose gel electrophoresis. Strain numbers in lanes: 1, B021; 2, 82.23.6; 3, IPVCT3C; 4, IPVCT4.1; 5, IPVCT2.2; 6, IPVCT7.2; 7, IPVCT3.1; 8, IPVCT5.1.t; 9, 60b; 10, IPVCTD1; 11, IPVCT5.2.t; 12, 632.2; 13, IPVCT8.3; 14, NCPPB2445; 15, IPVCT1.3; 16, IPVCT7.8; 17, B002; 18, J375; 19, IPVCTP1; 20, IPVCT4.5.t; 21, IPVCT4.2; 22, IPVCT4.4.t; 23, 1113.5; 24, control – no DNA; 25, IPVCT8.1; 26, IPVCTA1; 27, FPTPN CFBP 10435; 28, FPTPN CFBP1529; 29, FPTPN CFBP10530; 30, FPTPN 60; 31, *P. putida*; 32, *P. cichorii*; 33, *P. marginalis*; 34, *P. fluorescens*; 35, *P. syringae* pv. *syringae*; 36, *P. tolaasii*; M, 100 bp ladder.

Detection in infected tomato tissues

P. corrugata was successfully detected in artificially inoculated tomato plantlets affected by pith necrosis after amplification from a sample (5 μl) of DNA extracted by the quick alkaline method (Figure 7). No products were obtained from the control or from water-inoculated stem tissue. The quick alkaline method of DNA extraction with or without PVP on ground tissue was the most reproducible method, giving 100%

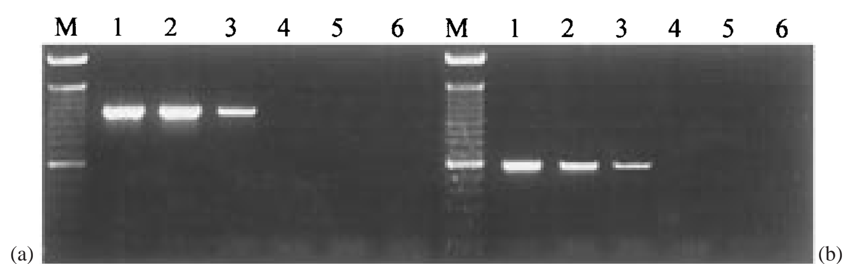

Figure 5. Sensitivity of detection in broth cultures with type I and type II primers. PCR amplification following serial dilution of broth cultures of *P. corrugata* (a) IPVCT10.3 (10^0 suspension = 10^9 cells ml^{-1}) and (b) IPVCT9.1 (10^0 suspension = 10^9 cells ml^{-1}). Lanes: 1, 10^{-1}; 2, 10^{-2}; 3, 10^{-3}; 4, 10^{-4}; 5, 10^{-5}; 6, no cells; M, 100 bp ladder.

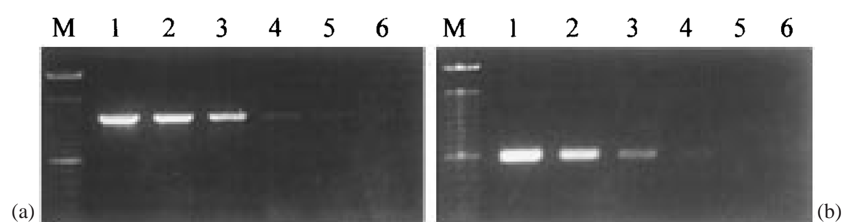

Figure 6. Sensitivity of detection from DNA with type I and type II primers. PCR amplification from isolated total DNA from *P. corrugata* (a) IPVCT10.3 and (b) IPVCT9.1. Lanes: 1, 20 ng; 2, 2 ng; 3, 0.2 ng; 4, 20 pg; 5, 2 pg; 6, 0.2 pg; M, 100 bp ladder.

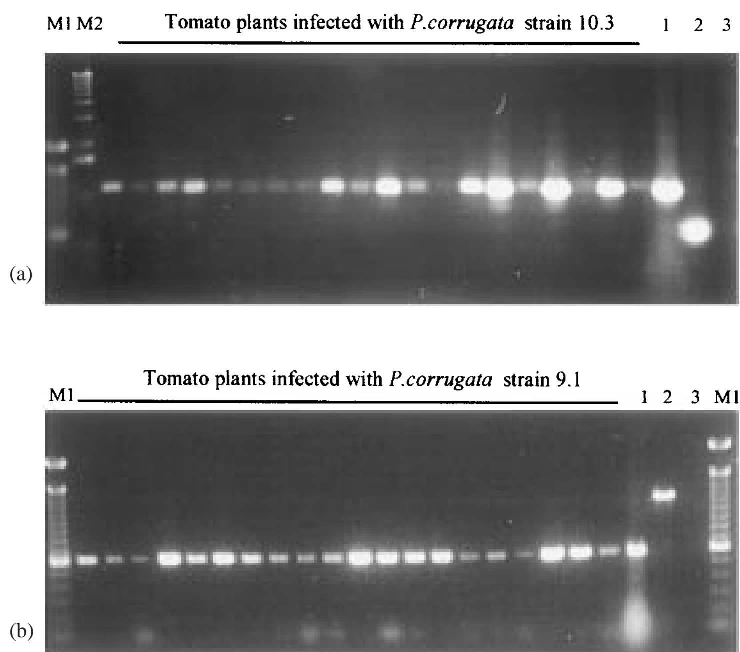

Figure 7. Detection of *P. corrugata* in infected tomato plants. DNA from tomato tissue, previously inoculated with different *P. corrugata* strains IPVCT10.3 (a) and IPVCT9.1 (b), was extracted by alkaline lysis and samples were PCR amplified with type I and type II primers in multiplex PCR reactions. Lanes: 1, IPVCT10.3 cells; 2, IPVCT9.1 cells; 3, water control. M1, 100 bp ladder; M2, 1 kb ladder.

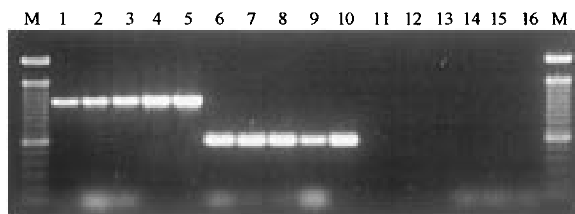

Figure 8. PCR amplification from stored lysates of infected tomato tissue. Alkaline lysates from tomato tissue infected with *P. corrugata* strains IPVCT10.3 (lanes 1–5) and IPVCT9.1 (lanes 6–10) and uninfected plants (11–15) stored at −20 °C and tested one month later by PCR amplification with type I and type II primers. Lane 16, water control; M, 100 bp ladder.

positive results from 50 infected plants. Positive results with the specific PCR primers were obtained from tissue stored at −20 °C for up to one month (Figure 8).

Discussion

A novel and rapid method for the identification and detection of *P. corrugata* in TPN disease is described. This bacterium is currently identified by biochemical tests and pathogenicity assays on tomato plants (Scarlett et al., 1978; Sutra et al., 1997). The use of serology and fatty acid methyl ester (FAME) profiles for identification and routine diagnosis of *P. corrugata* is limited by the high variability observed in this species (Siverio et al., 1993; 1996). Accurate identification of *P. corrugata* is important since symptoms of pith necrosis on tomato have also been ascribed to fluorescent *Pseudomonas* strains isolated from tomato pith necrosis in different countries (Dhanvantari, 1990; Catara et al., 1997; Sutra et al., 1997), *Erwinia* spp. (Victoria and Granada, 1983; Alivizatos, 1985; Dhanvantari and Duks, 1987) and other *Pseudomonas* spp. such as *P. cichorii* (Wilkie and Dye, 1974), *P. viridiflava* (Alivizatos, 1984) and *P. fluorescens* (Skoudridakis, 1986). The emended description of *P. corrugata* sets out the biochemical tests useful for its characterisation and identification (Sutra et al., 1997).

The use of two pairs of specific oligonucleotide primers with PCR amplification from bacterial cells or DNA provides a means of distinguishing *P. corrugata* from other related TPN-associated bacteria and enables detection in TPN lesions. The four primers, when used in multiplex PCR amplification from extracted total genomic DNA, with *P. corrugata* cells or with nucleic acids extracted from plants showing TPN, gave specific products of either 600 or 1100 bp. All *P. corrugata* strains tested were identified by the presence of one of the two PCR bands.

Many of the *P. corrugata* strains studied were previously characterised for their physiological characteristics, nutritional profile, IAA production, serological reactions and whole cell protein profiles. Despite the variability observed among strains, the phenotypic characteristics of *P. corrugata* clearly distinguished this group from other species (Catara et al., 1997). These *P. corrugata* strains formed three groups on the basis of the cluster analysis of nutritional profiles, but two groups on the basis of whole cell protein profiles by SDS-PAGE (Catara et al., 1997). Type I strains described here, which include the type strain, correspond to strains present in groups 2 and 3 of the cluster analysis of nutritional profiles and to a single group on the basis of SDS-PAGE profiles of whole cell proteins, while type II strains correspond to group 1 in the cluster analysis and to a single group on the basis of protein profiling (Catara et al., 1997).

Fragments amplified from total genomic DNA by RAPD-PCR appeared to show a major difference among the strains of *P. corrugata*. All strains produced either a type I band or a type II band with PCR, indicating the presence of at least two genomic groups. Genomic variability among the *P. corrugata* strains was also evident in the range of DNA reassociation levels that were observed (66–100%) by Sutra et al. (1997). Nevertheless, it was not strictly correlated with phenotypic clustering, where two subphena, designated 1a and 1b, were separated on the basis of three differential biochemical tests (Sutra et al., 1997). We included a number of *P. corrugata* strains from CFBP and the results show that the genomic groups I and II correspond, respectively, to the subphena 1a and 1b of Sutra et al. (1997). The genetic groups were not correlated with differences in pathogenicity or virulence of the strains. Bearing in mind that the number of strains examined was very small, group II appeared to be confined to Europe, since the four strains from other parts of the world were assigned to group I. The PCR limit of detection of *P. corrugata* was approximately 2–4 × 10^3 cfu ml^{-1} or about 20 pg of purified DNA. Similar detection limits were described for RAPD-PCR-based specific primers for the identification of *P. syringae* pv. *pisi* and *Xanthomonas fragariae* (Arnold et al., 1996; Pooler et al., 1996). The level of sensitivity could be a reflection of the high annealing

temperature used in the PCR assay or the fact that these primer pairs amplify single- or low-copy-number target sequences.

The development of this specific PCR protocol offers some distinct advantages over other methods for the identification of *P. corrugata*. Furthermore, it was successfully used to detect *P. corrugata* in artificially infected tomato plants showing the characteristic symptoms of the disease. The quick alkaline extraction protocol, previously used for the detection of common blight bacteria on bean leaves and common and halo blight bacteria in seed (Audy et al., 1994; 1996), was used for PCR detection of *P. corrugata* from necrotic lesions on tomato. The grinding step, although tedious, was necessary to obtain reliable results. Other extraction methods were not reproducible, probably due either to low levels or release of inhibitors from plant tissue when ground tissue was left to diffuse for a prolonged period (data not shown). No PCR products were obtained from TPN affected tomato plants, from which only fluorescent pseudomonads were obtained in culture (V. Catara, unpublished).

To determine the phylogeny of this bacterial species, it will be necessary to study the genomic diversity of *P. corrugata* by fingerprint techniques such as rep-PCR and restriction fragment length polymorphism analysis of PCR-amplified ribosomal operons and the internal transcribed spacer region. However, the PCR protocol described here offers an easy and quick method for the identification of *P. corrugata* strains. Its use for diagnostic purposes also provides a tool for epidemiological studies.

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