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The use of ARMS PCR in detection and identification of xanthomonads associated with pistachio dieback in Australia

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Abstract

Pistachio dieback occurs in the main pistachio growing areas of Australia. *Xanthomonas* strains belonging to the *translucens* group have been identified as the causal agent of the disease and two distinct groups, A and B, have been recognised within the pathogen population. In this study, specific primers for amplification of DNA of the pathogen were developed by sequencing the Internal Transcribed Spacer (ITS) region of rDNA from strains representing groups A and B, as well as from *X. translucens* isolated from wheat in Australia and one *Xanthomonas translucens* strain from orchard floor grasses. Primers were designed for amplification of DNA sequences specific to each group and a multiplex PCR test was developed that identified and differentiated strains of each group in a single PCR assay. To determine the specificity of the primers, PCR was carried out with DNA from 65 strains of the pistachio pathogen, 31 type and reference strains of *Xanthomonas*, and from 191 phytobacteria commonly found in and around pistachio orchards. In the multiplex PCR, a 331 bp fragment was amplified from all strains belonging to group A and a 120 bp fragment from all strains in group B. No PCR products were obtained from the other bacteria tested except for the type strain of *X. translucens* pv. *cerealis*, which has not been found in Australia. The assay was used to detect strains from both groups of the pathogen in pistachio plant material.

Introduction

Pistachio dieback occurs in the main pistachio growing areas of Australia and has not been reported elsewhere in the world. Discolouration of woody tissue in shoots one or more years old, trunk and limb lesions with excessive exudation of resin, and dieback and death of the tree characterise the disease. *Xanthomonas* strains have been identified as the causal agent of the disease and previous studies have shown a close relationship between the pathogen and *X. translucens* (Facelli et al., 2002, 2005; Marefat et al., 2006). The main location of the pathogen is in the young sapwood of the trunk, primary branches and young branches of diseased trees (Facelli et al., 2003) and the pathogen can be isolated most reliably from woody tissues with internal staining. We have demonstrated that two distinct phenotypic, genotypic and pathogenic groups, designated group A and B, are associated with the disease. Representatives of groups A and B have been accessioned in the International Collection of Micro-organisms from Plants, Auckland, New

Zealand, as ICMP 16316 and ICMP 16317, respectively (Marefat et al., 2006).

Group B strains have been reported from one pistachio growing area only, whereas group A strains are more widely distributed. There are no reports of coincident infection of trees by both groups. Strains belonging to group A appear to be more aggressive than group B and may pose more of a threat to the pistachio industry (Marefat et al., 2006).

While pistachio dieback is considered to be a serious threat to pistachio orchards in Australia (Anon., 2002), an efficient and reliable method to identify the pathogen in pure culture or plant material was lacking. Furthermore, the presence of the two groups within the pistachio pathogen has complicated the detection of the pathogen. Routine disease diagnosis currently depends upon the isolation of bacteria from infected woody tissues on agar media followed by physiological and biochemical tests to distinguish the pathogen from other bacteria and to identify it to group level. This process is laborious, unreliable and time-consuming, often requiring several weeks to complete. Therefore, the goal of this study was to develop a molecular PCR-based method that could detect and distinguish the two groups of the pathogen. Detection of the pathogen in plant material, especially asymptomatic infected plants, would assist in minimising spread of the disease.

Polymerase chain reaction (PCR)-based methods are being used increasingly for the detection and diagnosis of phytopathogenic bacteria and have proved to be fast, sensitive and reliable tools (Louws et al., 1999). A primer set has been reported for specific detection of *X. translucens* using PCR (Maes et al., 1996b). These primers, T1 and T2, were designed using ITS sequences to detect *Xanthomonas* pathogens that cause cereal leaf streak in seed. However, the assay recognises 10 pathovars of *X. translucens* and does not distinguish between them. In addition, *Xanthomonas* strains isolated from pistachio differ from *X. translucens* in certain phenotypic, genotypic and pathogenic features (Marefat et al., 2006; therefore methods used for *X. translucens* associated with cereals may be unsuitable for the specific detection of the pistachio pathogens.

The ribosomal DNA (rDNA) gene region is controlled by strong evolutionary and functional constraints and includes highly conserved regions (Fox et al., 1977; Gurtler and Stanisich, 1996), so it has been used as target for amplification in PCR-based techniques (Honeycutt et al., 1995; Maes et al., 1996a; Goncalves and Rosato, 2000). Although the 16S rDNA sequence has proved useful for the delineation of phytopathogenic bacteria at the genus level (DeParasis and Roth, 1990), variability has been found in the 16S–23S rDNA intergenic region which facilitates the development of species-specific primers (Barry et al., 1991). In particular, phylogenetic analysis of 16S and 16S–23S rDNA sequences of *Xanthomonas* species has revealed more variation in the ITS fragment than in the 16S rDNA (Hauben et al., 1997; Goncalves and Rosato, 2002) and this sequence has been targeted to design PCR primers specific for various *Xanthomonas* species (Maes et al., 1996b; Pan et al., 1997; Adachi and Oku, 2000).

Previously, we showed that the 16S–23S rDNA sequences of strains in group A and group B of the pistachio dieback pathogen were identical within each group and that the two groups differed in terms of a few bases in this region which could be targeted for the design of specific primers (Marefat et al., 2006).

The objectives of this study were to (i) evaluate *X. translucens*-specific primers in PCR for the identification of the pistachio dieback pathogen and, subsequently, to (ii) develop a PCR protocol for the detection of the pathogen that can differentiate the two groups within the population.

Materials and methods

Bacterial strains and growth

Bacterial strains used in this study and their sources are shown in Table 1. Sixty-five strains of the pistachio pathogen were obtained as described by Marefat et al. (2006). Thirty reference strains of the genus, including type strains, were obtained from the Belgium Coordinated Collection of Microorganisms, Laboratory of Microbiology, University of Ghent, Belgium (BCCM/LMG). *Xanthomonas albilineans* (ACM 1733) was obtained from the Australian Collection of Microorganisms (ACM), University of Queensland, Brisbane, Qld, Australia. *Xanthomonas translucens* pv. *translucens* (DAR 35705) from wheat, *X. axonopodis* pv. *malvacearum* (DAR 26904) and *X.*

Table 1. List of bacterial strains used in this study, results of PCR using the *Xanthomonas translucens*-specific primer set, T1 and T2, and primers designed in this study

Strains and accession numbers	Source	PCR[a]		
		T1 and T2	PAf and PABr	PBf and PABr
Pistachio pathogen strains, group A (2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14, 15, 16, 20, 28, 29, 30, 31, 32, 33, 34, 72, 73, 74, 75, 76, 77, 78, ICMP 16316)	NT99004[b] NT02007[c]	+	+	−
Pistachio pathogen strains, group B (21, 22, 23, 24, 26, 27, 36, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, ICMP 16317)	NT99004 NT02007	+	−	+
Xanthomonas translucens pv. *poae* (LMG 728)	BCCM[d]	+	−	−
X. translucens pv. *phleipratensis* (LMG 843)	BCCM	+	−	−
X. translucens pv. *hordei* (LMG 737)	BCCM	+	−	−
X. translucens pv. *phlei* (LMG 730)	BCCM	+	−	−
X. translucens pv. *undulosa* (LMG 892)	BCCM	+	−	−
X. translucens pv. *translucens* (LMG 876)	BCCM	+	−	−
X. translucens pv. *cerealis* (LMG 679)	BCCM	+	−	+
X. translucens pv. *secalis* (LMG 883)	BCCM	+	−	−
X. translucens pv. *graminis* (LMG 726)	BCCM	+	−	−
X. translucens pv. *arrhenatheri* (LMG 727)	BCCM	+	−	−
X. pisi (LMG 847)	BCCM	N	−	−
X. vasicola pv. *holcicola* (LMG 936)	BCCM	N	−	−
X. campestris pv. *campestris* (LMG 568)	BCCM	N	−	−
X. arboricola pv. *corylina* (LMG 689)	BCCM	N	−	−
X. arboricola pv. *juglandis* (LMG 747)	BCCM	N	−	−
X. arboricola pv. *pruni* (LMG 852)	BCCM	N	−	−
X. hortorum pv. *hederae* (LMG 733)	BCCM	N	−	−
X. oryzae pv. *oryzae* (LMG 5047)	BCCM	N	−	−
X. theicola (LMG 8684)	BCCM	N	−	−
X. fragariae (LMG 708)	BCCM	N	−	−
X. cassavae (LMG 673)	BCCM	N	−	−
X. axonopodis pv. *axonopodis* (LMG 982)	BCCM	N	−	−
X. codiaei (LMG 8678)	BCCM	N	−	−
X. sacchari (LMG 471)	BCCM	N	−	−
X. bromi (LMG 947)	BCCM	N	−	−
X. hyacinthi (LMG 739)	BCCM	N	−	−
X. melonis (LMG 8670)	BCCM	N	−	−
X. cucurbitae (LMG 690)	BCCM	N	−	−
X. vesicatoria (LMG 911)	BCCM	N	−	−
X. sp. pv. *mangiferaeindicae* (LMG 941)	BCCM	N	−	−
X. albilineans (ACM 1733)	ACM[e]	N	−	−
X. translucens pv. *translucens* (DAR 35705)	ACPPB[f]	+	−	−
X. arboricola pv. *pruni* (DAR 64858)	ACPPB	−	−	−
X. axonopodis pv. *malvacearum* (DAR 26904)	ACPPB	−	−	−
191 phytobacteria isolated from plants in and around pistachio orchards	NT02007	15[g]	−	−

[a]Detection of bacteria with T1 and T2: specific primers for *X. translucens* (Maes et al., 1996b), and PAf and PABr and PBf and PABr: primers designed in this study. +: amplicon of expected size obtained, −: no amplicon and N: not determined.
[b]Pistachio canker epidemiology, Project NT99004, Horticulture Australia Limited.
[c]Molecular techniques to identify and detect the bacteria associated with pistachio dieback, Project NT02007, Horticulture Australia Limited.
[d]BCCM: Belgium Coordinated Collection of Microorganisms, Laboratory of Microbiology, University of Ghent, Belgium.
[e]ACM: Australian Collection of Microorganisms, University of Queensland, Brisbane, Qld, Australia.
[f]ACPPB: Australian Collection of Plant Pathogenic Bacteria, Agricultural Institute, Orange, NSW, Australia.
[g]The number of bacterial strains detected. These 15 *X. translucens*-like strains had the same features in biochemical tests and showed similar rep-PCR fingerprints (data not shown). Strain 61W was selected as representative of this group for further study.

arboricola pv. *pruni* (DAR 64858) from stone fruits, all isolated in Australia, were obtained from the Australian Collection of Plant Pathogenic Bacteria (ACPPB), Agricultural Institute, Orange, NSW, Australia.

To assess the specificity of DNA-based detection methods for the pathogen, other bacteria occurring naturally in and around pistachio orchards were collected. Two orchards were selected in two major pistachio-growing areas, Kyalite, NSW in which group A is known to occur and Robinvale, South Australia from which only group B has been isolated. Samples were collected from apparently healthy pistachio tissues (leaf, shoot, bud and bark), from grasses, weeds and other plants in the orchards and from plant species in the vicinity of the orchards in the autumn (May), winter (August) and summer (December). Plant material was washed in 200 ml sterile water containing a drop of Tween 20 wetting agent on a shaker for 20 min, then 100 μl of the suspension was streaked on nutrient agar (NA), nutrient agar plus sucrose (NAS) and King's B medium (Schaad et al., 2001). All plates were incubated at 25 °C for 72 h and 191 representative bacteria with various morphological characteristics were selected.

Freeze-dried strains, obtained from the culture collections, were revived on yeast dextrose carbonate (YDC) agar at 28 °C (Schaad et al., 2001). Strains were grown on NA and YDC at 28 °C for 3 days for use in subsequent tests. All bacteria were routinely stored on NA and YDC and frozen in 30% glycerol at −70 °C for long-term preservation.

Preparation of DNA

All isolates listed in Table 1 were grown on tryptone soy agar (TSA) (15 g tryptone, 5 g soy peptone, 5 g NaCl, 15 g agar in 1000 ml water, pH 7.3) at 28 °C for 48 h and the genomic DNA was extracted following the method of Rademaker and de Bruijn (1997). DNA concentration was determined by optical density (260 nm) and diluted to 50 ng μl^{-1}.

Detection of bacteria with Xanthomonas translucens*-specific primers, T1 and T2*

Xanthomonas translucens-specific primers, T1 and T2 (Maes et al., 1996b), were tested to determine if they could be used for the specific amplification of the pistachio pathogen. DNA from all pathogen strains and phytobacteria isolated from plants in and around pistachio orchards was tested. *Xanthomonas translucens* pv. *translucens* DAR 35705 was used as a positive control and *X. axonopodis* pv. *malvacearum* (DAR 26904) and *X. arboricola* pv. *pruni* (DAR 64858) were used as negative controls. Primers were synthesized by Proligo (Proligo Pty Ltd, Lismore, Australia) and PCR amplification was performed on a Peltier Thermal Cycler model PTC-200 (MJ Research Inc., CA, USA), as described by Maes et al. (1996b) with minor modification. The reaction mixture was prepared in a total volume of 20 μl comprising 0.2 μl *Taq* polymerase (5 U μl^{-1}, Invitrogen Pty Ltd, Victoria, Australia), 2 μl 10 × PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl), 0.6 μl $MgCl_2$ (50 mM), 0.16 μl dNTP mixture (25 mM each), 0.8 and 0.6 μl of T1 and T2 primers (10 μM each), respectively, 1 μl sample (50 ng μl^{-1}) and 14.64 μl distilled water. PCR was performed with the following conditions: 1 × 90 °C for 2 min, 35 × (93 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min) and 72 °C for 10 min. The PCR products were analysed by electrophoresis on a 2% agarose gel at 10 V cm^{-1} for 70 min, staining with ethidium bromide and visualising under UV light.

Sequencing of the 16S–23S rDNA spacer region

Seven strains of the pathogen, four from group A (strains 3, 28, 30 and ICMP 16316) and three from group B (strains 36, 50 and ICMP 16317), along with *X. translucens* pv. *translucens* DAR 35705 were selected for sequencing of the 16S–23S rDNA spacer region. One *X. translucens*-like bacterium (strain 61W) was included in the sequencing as the representative of 15 bacteria obtained from orchard floor grasses and detected by the T1 and T2 primer set. These 15 strains had the same features in biochemical tests and showed similar patterns in rep-PCR fingerprinting (data not shown). The fragment was first amplified in PCR using a forward 20-bp primer, 5′-AGT CGT AAC AAG GTA AGC CG-3′ (C1), derived from the *Escherichia coli* 16S rDNA position 1493–1513, and a reverse 20-bp primer, 5′-C(T/C)(A/G) (T/C)TG CCA AGG CAT CCA CC-3′ (C2), corresponding to the *E. coli* 23S rDNA sequence position 23–43 (Lane, 1991). Reaction conditions for a 50-μl PCR

were: 0.5 μl *Taq* polymerase (5 U μl^{-1}, Qiagen Pty Ltd, Victoria, Australia), 5 μl 10 × PCR buffer, 0.4 μl dNTP mixture (25 mM each), 2.5 μl of C1 and C2 primers (10 μM each), 1 μl sample (50 ng μl^{-1}) and 38.1 μl distilled water. PCR was performed as above with the following conditions: 1 × 95 °C for 15 min, 29 × (95 °C for 45 s, 50 °C for 1 min, 72 °C for 2 min) and 72 °C for 10 min. The PCR product (10 μl) was run on an agarose gel (1%) and stained with ethidium bromide. The Perfectprep® Gel Cleanup Kit (Brinkmann Instruments, Inc., NY) was used to purify the fragment from the gel as recommended by the manufacturer. DNA sequencing was performed in both directions using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, CA). The sequencing reaction was precipitated using isopropanol and analysed on an ABI Prism 3700 DNA analyser at the Sequencing Centre, Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. The Chromas (version 2) software (Technelysium Pty Ltd, Qld, Australia) was used for editing and generating the sequences. Alignment and comparison of the sequences were performed with programmes Clustal X and GeneDoc available on the Bioinformatics.Net database. Using the Basic Local Alignment Search Tool (BLAST), the sequences were compared with sequences in the GenBank, EMBL, DDBJ and PDB databases. To check reproducibility, samples were prepared for sequencing twice, each time from different DNA preparations.

Selection and design of PCR primers specific for the pathogen

Sequences in the ITS which diverged between group A and group B strains, and between these two groups and other xanthomonads, were identified as sites for the design of primers. Primers were designed for specific identification of groups A and B and also for use in a multiplex PCR, in which two pairs of primers are used simultaneously, to distinguish groups A and B from one another and from other bacteria in a single assay. Some primers were designed based on the Amplification Refractory Mutation System (ARMS) (Newton et al., 1989), in which a primer is designed so that it can discriminate between templates which differ in a specific single nucleotide. The oligonucleotides were synthesised by GeneWorks Pty Ltd (Adelaide, Australia). The suitability of the primers was assessed using DNA from representative strains from the two groups of the pathogen, type and reference strains from the genus, *X. translucens* pv. *translucens* DAR 35705 and the *X. translucens*-like bacterium strain 61W isolated from grasses in pistachio orchards as a template in the PCR. Finally, two forward primers, hereafter named PAf and PBf, and one reverse primer, namely PABr, were developed to distinguish groups A and B strains from one another and from other bacteria in a single assay (Table 2). The comparison of these primers with DNA sequences in the GenBank, EMBL, DDBJ and PDB databases, using the BLAST programme, suggested that the primers would be highly specific for pistachio pathogen strains.

PCR cycling conditions

For each standard PCR, 0.25 μl of DNA (50 ng μl^{-1}) was added to a final volume of 25 μl containing 0.1 μl *Taq* polymerase (5 U μl^{-1}), 2.5 μl 10 × PCR buffer, 0.2 μl dNTP mixture (25 mM each), 1.25 μl of the reverse (PABr) and the forward (PAf or PBf) primers (10 μM each), and 19.45 μl distilled water. The reaction was carried out in a Peltier Thermal Cycler as above with the following programme: 1 × 95 °C for 15 min, 30 × [94 °C for 45 s, 53 °C (for primers PAf and PABr) or 56 °C (for primers PBf and PABr) for 45 s, 72 °C for 2 min] and 72 °C for 10 min. For the multiplex PCR, 1.25, 1.5 and 1 μl of PABr, PAf and PBf primers (10 μM each), respectively, were added to the other components as above and the final volume was adjusted to 25 μl with distilled water. The annealing temperature was adjusted to 54 °C. Six μl of the PCR product was subjected to electrophoresis on an agarose gel (2%) using Tris–acetate EDTA (TAE) buffer at 10 V cm^{-1} for 70 min, stained with ethidium bromide and visualized under UV light.

Table 2. Oligonucleotide primers designed based on the 16S–23S rDNA sequencing data shown in Figure 2, for specific detection of pistachio dieback pathogens

PAf	5′-CCTCCTTTTGAGCATGAGAA-3′
PBf	5′-ACAGTCTAAGGGACCTGCG-3′
PABr	5′-TCACTGCTGGCGCATCTTA-3′

PCR specificity and sensitivity tests

To assess the specificity of the designed primers, the multiplex PCR was carried out with DNA from the 65 *Xanthomonas* strains from pistachio and other bacteria listed in Table 1. To determine the detection limit of the primers, 10-fold serial dilutions were made from strains ICMP 16316 and ICMP 16317, and 5-μl aliquots from each dilution were added directly to each standard PCR mixture. The number of cells in each series was assessed by counting colony forming units (CFU) following plating on YDC agar medium. This assessment was performed for each strain individually and repeated three times.

PCR efficiency in plant material and verification of PCR results

Two assays were performed to evaluate detection of pathogens in plant material. In the first assay, shoot samples from trees which had been artificially infected with the pathogen strains from group A and group B, individually, 20 months previously and that showed internal staining characteristic of the disease were used. For the second assay, shoot samples, 2–3 years old, were collected from symptomatic and asymptomatic trees in pistachio orchards naturally infected by group A (Kyalite) or B (Robinvale). Shoot samples were also collected from a healthy tree in a non-infected area as a negative control. Woody tissue (500–750 mg) was removed from the samples and soaked in 10 ml of nutrient broth overnight in a shaking-incubator at 27 °C. The resulting suspension was transferred to a fresh tube and centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 100 μl sterile distilled water and a 5-μl aliquot was used as PCR template. To adsorb polyphenolic materials that can inhibit the PCR, polyvinylpyrrolidone (PVP, MWt 44,000) was added to each reaction mixture (1% final concentration) (Fegan et al., 1998; Koonjul et al., 1999). Also, the remaining suspension (95 μl) was plated on YDC to confirm the presence/absence of the pathogen. In the case of uncertain colony identification, the identity of the recovered bacteria was confirmed by comparing them with representatives of groups A and B using BOX-PCR (Rademaker and de Bruijn, 1997; Marefat et al., 2006).

Results

Detection of bacteria with X. translucens*-specific primers, T1 and T2*

A 139-bp fragment, specific for *X. translucens* pathovars (Maes et al., 1996b), was amplified using the published primers, T1 and T2, from the 65 *Xanthomonas* strains from pistachio, belonging to both group A and B, as well as from *X. translucens* pv. *translucens* DAR 35705, the positive control, and 15 *X. translucens*-like bacteria isolated from grasses in pistachio orchards. In contrast, no PCR amplicon was obtained from the negative controls comprising DNA from *X. axonopodis* pv. *malvacearum* (DAR 26904) and *X. arboricola* pv. *pruni* (DAR 64858) (Figure 1).

The 16S–23S rDNA sequence data

The complete 16S–23S rDNA spacer region was sequenced for seven *Xanthomonas* isolated from pistachio (strains 3, 28, 30 and ICMP 16316 from group A; and 36, 50 and ICMP 16317 from group B). A fragment of 558 bases was sequenced for all four strains representing group A and a fragment of 557 bases for all three strains representing group B. No sequence differences were observed among strains of each group. However, the two groups differed in 1, 6 and 5 bases in ITS1, ITS2 and ITS3, respectively. Alignment of the sequences with

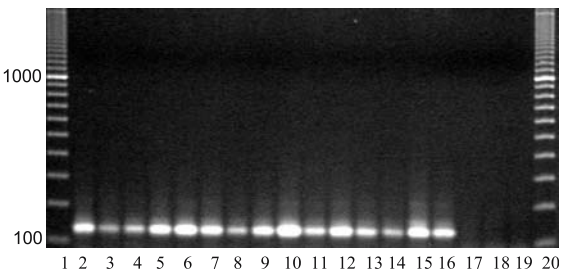


Figure 1. Electrophoretic analysis of PCR-amplified DNA using *Xanthomonas translucens*-specific primers, T1 and T2 (Maes et al., 1996b). Lanes: 1 and 20, molecular weight markers (bp) (100 bp ladder, Invitrogen, Australia); lanes 2–8, pistachio pathogen strains from group A; 9–14 pathogen strains from group B; lane 15, *X. translucens* pv. *translucens* (DAR 35705); lane 16, *X. translucens* strain 61W isolated from pistachio orchard grasses; lane 17, *X. axonopodis* pv. *malvacearum* (DAR 26904); lane 18, *X. arboricola* pv. *pruni* (DAR 64858) and lane 19, the PCR mixture without DNA.

sequences obtained from *X. translucens* pv. *translucens* DAR 35705 and *X. translucens* 61W isolated from grasses in pistachio orchards in this study, and from *X. translucens* pv. *graminis* and *X. translucens* pv. *poae*, sequences of two closely related bacteria in the GenBank database, is shown in Figure 2. The nucleotide sequences determined in this study have been deposited in the Genbank database under the following accession numbers. AY579378: pistachio pathogen strain ICMP 16316 representative of group A, AY579379: pistachio pathogen strain ICMP 16317 representative of group B, AY994098: *X. translucens* pv. *translucens* DAR 35705, isolated from wheat in Australia, and AY994099: *X. translucens* 61W isolated from grasses in pistachio orchards in Australia.

Selection and design of PCR primers

Two forward primers, PAf and PBf, specific for group A and group B, respectively, and PABr, a single reverse primer specific to the sequence conserved between the two groups, were selected (Figure 2, Table 2). PAf, an ARMS primer (Newton et al., 1989), is complementary to the corresponding sequence of the group-A pathogen strains except for one additional deliberate mismatch, G/C, at the third nucleotide from the 3′-OH terminus of the primer. However, there are two mismatched nucleotides, A/G at the 3′-end and G/C three bases from the 3′-end, between this primer and the corresponding sequence of the other bacteria, including group B. The deliberate mismatch was introduced to enhance discrimination between group A strains and other bacteria. Using the primers, a multiplex PCR assay was successfully developed to amplify a 331-bp group A-specific DNA fragment and a 120-bp group B-specific DNA fragment in a single test.

PCR specificity and sensitivity tests

All 29 strains of the pathogen belonging to group A produced the unique amplification product of 331 bp when DNA from these bacteria was used as a template for the multiplex PCR with the designed primers, and all 36 strains of the pathogen belonging to group B produced the unique product of 120 bp. In contrast, no PCR products were obtained from the other bacteria tested except for the type strain of *X. translucens* pv. *cerealis* (Figure 3). The minimum number of cells detected by the PCR was 30 per each standard reaction (Figure 4).

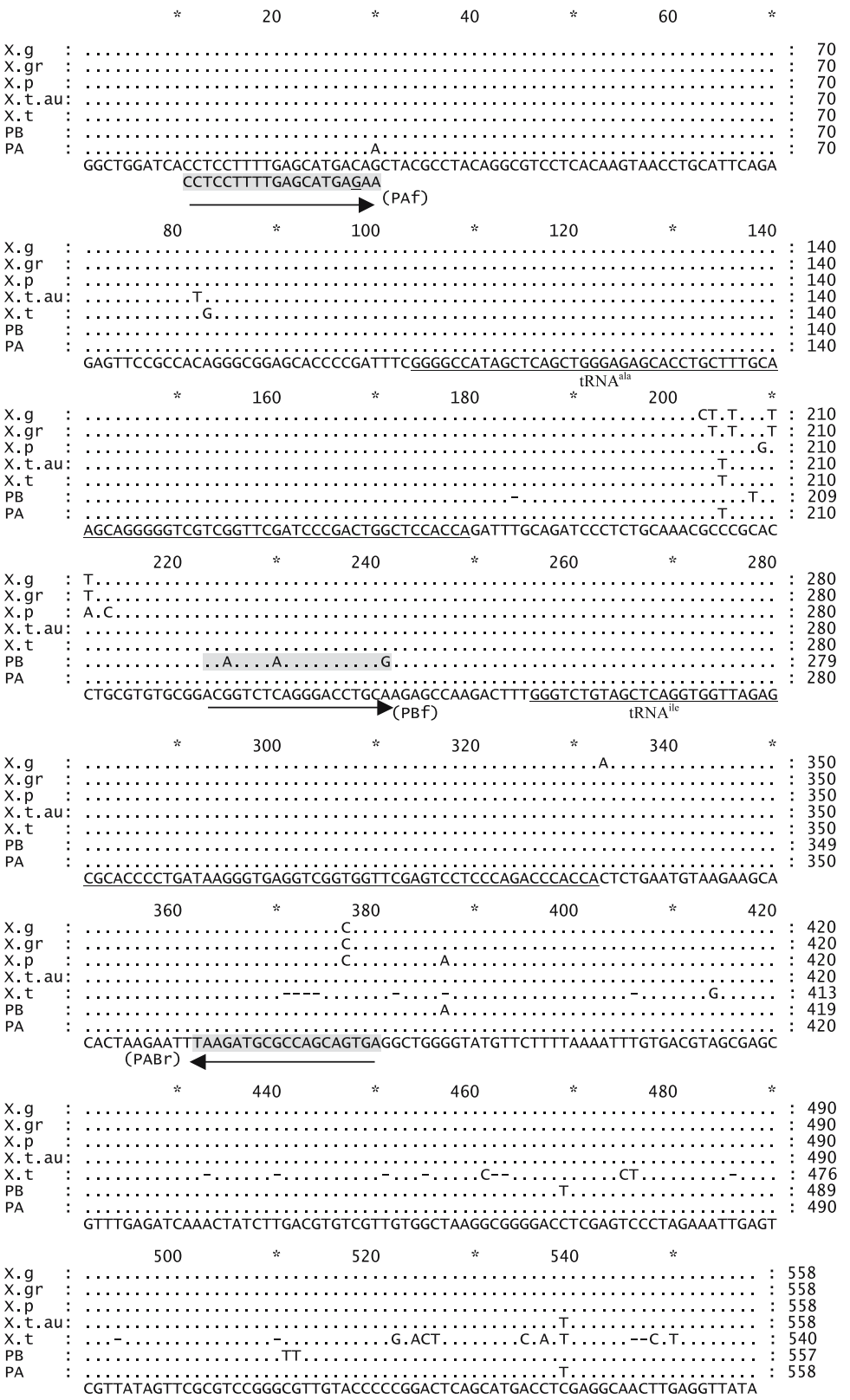
PCR efficiency in plant material

The predicted PCR product was amplified from all symptomatic samples of pistachio wood from both artificially and naturally infected trees, as well as from some asymptomatic samples collected from both artificially and naturally infected pistachio shoots. These products were detectable only when PVP was added to the PCR. No amplified product was detectable from a mixture comprising plant tissue and the pathogen genomic DNA without PVP, indicating that there was a noticeable inhibition of the PCR by pistachio tissue. Also, no product was observed from the healthy plant tissue, and there were no non-specific PCR products. The pathogen strains from both groups were detected in pistachio plant material by the assay. The detection of the pathogen, belonging to group A, in a naturally infected pistachio sample is shown in Figure 5. From all plant samples that showed a positive signal in the assay, bacterial strains were recovered on culture medium and assigned to group A or B of the pathogen by BOX-PCR (data not shown).

Discussion

A rapid, sensitive and highly specific method has been developed for the identification of xanthomonad pathogens of pistachio in Australia. This method uses a unique primer design (ARMS) in a multiplex-PCR, can be used to detect and to identify the pathogen in culture and in plant material, and is able to differentiate between the two groups of the pathogen (A and B) simultaneously.

Although the PCR-based protocol developed by Maes et al. (1996b) for detection of *X. translucens* amplified the expected fragment of 139 bp from DNA of the pistachio strains, the assay could not distinguish groups A and B. Furthermore, PCR using T1 and T2 amplified a fragment of 139 bp from the 10 pathovars of *X. translucens* tested, all of which are known pathogens of cereals and grasses (Maes et al., 1996b), as well as from the *X. translucens*-like bacterium (strain 61W) which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AGCAGGGGGTCGTCGGTTCGATCCCGACTGGCTCCACCAGATTTGCAGATCCCTCTGCAAACGCCCGCAC
CTGCGTGTGCGGACGGTCTCAGGGACCTGCAAGAGCCAAGACTTTGGGTCTGTAGCTCAGGTGGTTAGAG
(PBf)
tRNA^ile
CGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCACTCTGAATGTAAGAAGCA
CACTAAGAATTTAAGATGCGCCAGCAGTGAGGCTGGGGTATGTTCTTTTAAAATTTGTGACGTAGCGAGC
(PABr)
GTTTGAGATCAAACTATCTTGACGTGTCGTTGTGGCTAAGGCGGGGACCTCGAGTCCCTAGAAATTGAGT
CGTTATAGTTCGCGTCCGGGCGTTGTACCCCCGGACTCAGCATGACCTCGAGGCAACTTGAGGTTATA

Figure 2. ITS sequences used to design primers specific for PCR amplification of pistachio dieback pathogen rDNA. X. g: *Xanthomonas translucens* pv. *graminis* (Genbank, AY247064.1), X. gr: *X. translucens* strain 61W isolated from pistachio orchard floor grasses (Genbank: AY994099), X. p: *X. translucens* pv. *poae* (Genbank: AY253329), X. t. au: *Xanthomonas t*. pv. *translucens* (DAR 35705) isolated from wheat in Australia (Genbank: AY994098), X. t: *Xanthomonas translucens* pv. *translucens* (Genbank: AF209764.1), PB: pistachio strain ICMP 16317 representative of group B (Genbank: AY579379) and PA: pistachio strain ICMP 16316 representative of group A (Genbank: AY579378). The conserved bases are denoted in the bottom line. Dash denotes deletion in the sequence. $tRNA^{ala}$ and $tRNA^{ile}$ genes are underlined. Sequences of the primers are shaded and arrows indicate the direction of priming of the primers. The ARMS primer used in this study is shown below the sequence and the underlined letter indicates the nucleotide alteration introduced to enhance the 3′ mismatch effect.

regularly has been isolated from grasses in pistachio orchards. Our study indicated that this *X. translucens* is genetically distinct from the pistachio pathogens and is a pathogen of grasses only (A. Marefat et al., unpublished data).

The sequence variation in the intergenic spacer flanked by 16S and 23S rRNA genes between pistachio dieback strains in group A and B and between these two groups and other xanthomonads studied, provided an opportunity to design specific primers for use in a multiplex PCR assay

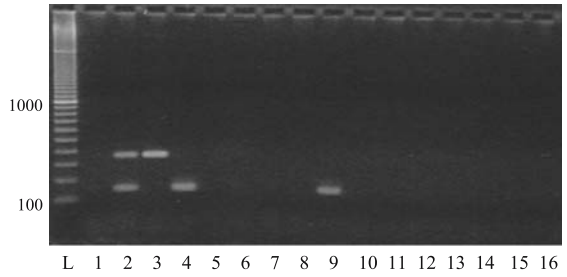

Figure 3. Specific amplification of target DNA from the pistachio pathogen by a multiplex PCR. Lanes: 1, control (no DNA); 2, a mixture of strains ICMP 16316 and ICMP 16317 representing groups A and B, respectively; 3, group A representative (ICMP 16316); 4, group B representative (ICMP 16317); 5, *X. translucens* (61W) isolated from grasses on pistachio orchard floor; 6, *X. t*. pv. *translucens* DAR 35705 isolated from wheat in Australia. 7, *X. t*. pv. *undulosa*; 8, *X. t*. pv. *translucens*; 9; *X. t*. pv. *cerealis*; 10, *X. t*. pv. *secalis*; 11, *X. t*. pv. *poae*, 12, *X. t*. pv. *phleipratensis;* 13, *X. t*. pv. *arrhenatheri*; 14. *X. t*. pv. *graminis*; 15, *X. t*. pv. *phlei*; 16, *X. t*. pv. *hordei*. L: molecular weight markers (bp) (100 bp ladder, Invitrogen, Australia).

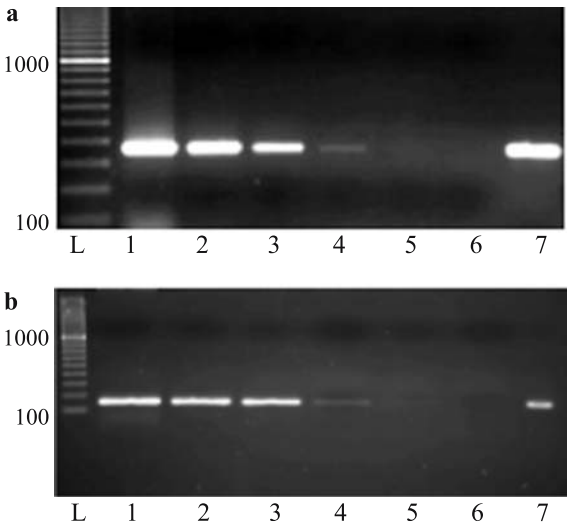

Figure 4. Agarose gel electrophoresis of standard PCR products from a dilution series of the pistachio dieback pathogen. a: Representative from group A (ICMP 16316), b: representative from group B (ICMP 16317). Lanes 1–5 are the PCR products from 3×10^4, 3×10^3, 3×10^2, 3×10^1 and 0 CFU. Lanes 6 and 7 are negative and positive controls, respectively. L: Molecular weight markers (bp) (100 bp ladder, Invitrogen, Australia).

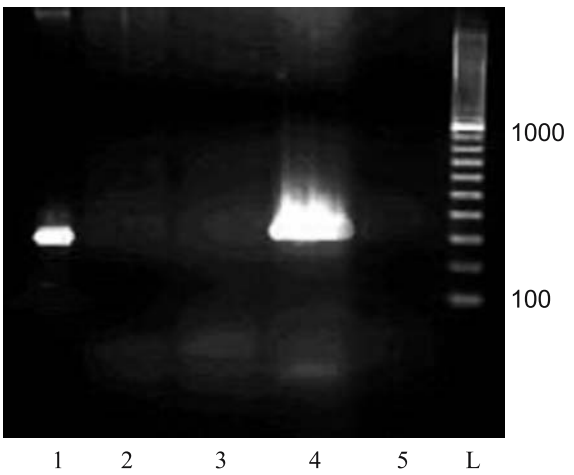

Figure 5. Detection of pistachio dieback pathogen, belonging to group A, in naturally infected plant material. Samples prepared for PCR by soaking pistachio woody tissue in nutrient broth overnight, centrifuging and resuspending the pellet in sterile distilled water. Lanes: 1, PCR mixture with pathogen genomic DNA (positive control); 2, mixture comprising infected plant tissue and the pathogen genomic DNA without PVP; 3, sample prepared from infected plant tissue without PVP; 4, sample prepared from infected plant tissue plus PVP (MWt 44,000, 1% final concentration); 5, sample prepared from a healthy plant (negative control).

for the detection of the pathogen strains from both groups in a single assay.

The Amplification Refractory Mutation System (ARMS) (Newton et al., 1989) for primer development was used to distinguish strains belonging to group A from other bacteria including group B. In ARMS-PCR, a primer is designed so that it can discriminate between templates which differ at a specific single nucleotide at the 3′-terminal base of the primer. The basis of the technique is that *Taq* polymerase lacks a 3′–5′ exonuclease activity; therefore oligonucleotides with a mismatched 3′-end cannot function as primers in the PCR. Although a single base mismatch located at the 3′-end of the primer is sufficient for preferential amplification of the perfectly matched oligonucleotide, incorporating an artificial mismatch at the residue 1 or 2 bases from the 3′-end of the primer can enhance primer specificity under appropriate conditions. This deliberate mismatch has no significant influence on the yield of PCR products, whereas the mismatch at the 3′-OH extremity of the primer is refractory to extension by the *Taq* DNA polymerase in order that the yield of product from the other bacteria is not detectable (Newton et al., 1989). The ARMS primer designed in this study, PAf, generates a deliberate mismatch (G/C, primer/sequence) three bases from the 3′-end with the corresponding sequence of strains belonging to group A. Besides this mismatch, PAf forms another mismatch (A/G, primer/sequence) at the 3′-end with other bacteria including group B. The type and the position for introduction of the deliberate mismatch was optimised by comparing results of several ARMS primers with various alterations (data not shown); of these, PAf gave the most effective discrimination. This confirms that a strong mismatch (purine/purine or pyrimidine/pyrimidine) at the 3′-terminus of a nucleotide-specific primer is likely to need a weak second mismatch (purine/pyrimidine) (Ye et al., 2001). ARMS-PCR is increasingly being applied in medicine and genetics to detect point mutations and deletions/insertions in the genomic DNA (Ye et al., 2001; Fan et al., 2003) and has recently been utilized for reliable differentiation between closely related plant pathogenic fungi (Jayne and Taylor, 2001; Mach et al., 2004). The study described here is thought to be the first description of the use of ARMS-PCR for the detection of a plant pathogenic bacterium.

In order to develop a multiplex-PCR to detect and distinguish both groups simultaneously, the specific forward primer for strains belonging to group B, PBf, was selected so that the primer annealing condition was roughly similar to that of PAf, and so that the amplified DNA fragments were of different length using a common reverse primer (PABr). The development of a multiplex-PCR to detect more than one target simultaneously needs extensive optimization (Henegariu et al., 1997). The best result for the triple primer pair multiplex-PCR designed in this study was achieved with optimization of primer concentration and annealing temperature which allows detection of either or both of two groups of the pathogen simultaneously. Multiplex-PCR has also been used by Fegan et al. (1998) and Glick et al. (2002) for concurrent detection of two phytopathogenic bacteria in other studies.

In developing a specific PCR test for a pathogen, specificity and sensitivity of the test are two important factors. The specificity of the assay reported here is adequate as the predicted products were amplified from all pathogen strains belonging to groups A and B, and no amplicons were obtained from the other bacteria isolated from plants in and around pistachio orchards or from type and reference strains of the genus, except for the type strain of *X. translucens* pv. *cerealis*. The latter has not been reported in Australia. In experiments performed on numerous plant samples, no non-specific PCR signal produced by plant material or other plant-associated organisms has been observed so far. Xanthomonads were recovered from plant samples which were positive in the PCR assay and their identity was confirmed by BOX-PCR (data not shown).

When testing plant material, specific amplification of target DNA was successful only when PVP was used in the PCR. Phenolic terpenoids and tannins present in plant material can bind to RNA and DNA upon cell lysis and the use of PVP has been proposed to purify nucleic acids in plant extracts (John, 1992). To minimize the effect of these compounds, which can inhibit the PCR, PVP has been used either during extraction of bacterial DNA from plant material (Maes et al., 1996a; Meng et al., 2004) or directly in the PCR (Fegan et al., 1998; Koonjul et al., 1999).

Specific bands were observed on ethidium bromide-stained agarose gel when using 30 bacterial

cells per PCR (25 μl). This sensitivity corresponds to an average detection limit of 1.2×10^3 CFU of the pathogen per ml. Conventional methods for the detection of plant pathogenic bacteria often use plating on agar media and serological techniques. Plating on medium has the potential to detect single, viable cells; however the percentage recovery of target bacteria from plant tissue depends on several factors, such as the optimal release of the target from tissue, the minimization of competition by saprophytic microbiota and the minimization of interference by inhibitory plant compounds and debris (Saettler et al., 1989). Indeed, isolation into culture is not enough to identify the pathogen and further tests are required, especially to distinguish the two groups. Labour costs and time are other disadvantages of the culture technique. Immunodiagnostic methods, such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF), both popular and successful formats, provide rapid detection and identification of bacteria; however the detection limit is normally around 1×10^5 CFU ml^{-1} (Alvarez, 2001). Furthermore, techniques based on polyclonal and monoclonal antibodies cannot differentiate among the various pathovars of *X. translucens* because of the high degree of serological similarities within this group (Azad and Schaad, 1988; Bragard and Verhoyen, 1993).

The PCR test developed in this study has several advantages, as it works well with genomic DNA, can be applied directly to colonies on agar media, and can be used directly on plant material to detect the pathogen and identify it to group level simultaneously. Detection of infection in plant material can be achieved within 1 day. The assay was able to detect the pathogen in some asymptomatic samples collected from artificially and naturally infected trees from which the pathogen was recovered on agar medium. Detection of the pathogen in plant material, especially in asymptomatic, infected planting material may be important in minimising the spread of the disease. Thus, this technique will contribute to an understanding of the epidemiology of the disease in order to reduce risk.

Sequence differences, in the ITS, between pistachio strains and some other pathovars of the *translucens* group as well as our unpublished data confirm that the pistachio pathogen is different from known pathovars of *X. translucens*. A comprehensive polyphasic study to determine the exact situation of the pathogen within the species is in progress.

In summary, a PCR-based assay based on the 16S–23S internal spacer region was developed for identification of the xanthomonad pathogens of pistachio in Australia. The assay was successfully used to detect the pathogen in culture and in plant material. Furthermore, the two groups of the pathogen (A and B) could be distinguished in a multiplex PCR. This assay is now being used in epidemiological studies of pistachio dieback and will provide the pistachio industry with a powerful and specific molecular tool for the detection of the dieback pathogens, thereby contributing to disease management.

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