Asia Pacific Journal of Molecular Biology and Biotechnology
Volume 5, No. 1, April, 1997 pp. 19-30.

A Novel Method For Development Of Species And Strain-Specific DNA Probes And PCR Primers For Identifying *Burkholderia Solanacearum* (Formerly *Pseudomonas Solanacearum*)

N. OPINA[1,2], F. TAVNER[1], G. HOLLWAY[1], J.-F. WANG[3], T.- H. LI[3], R. MAGHIRANG[2,3], M. FEGAN[4], A. C. HAYWARD[4], V. KRISHNAPILLAI[5], W. F. HONG[5], B. W. HOLLOWAY[5] and J . N. TIMMIS[1]*

[1]*Department of Genetics, The University of Adelaide, South Australia 5005, Australia,* [2]*Present address: Institute of Plant Breeding, University of The Philippines, Los Banos, 4031, The Philippines,* [3]*Asian Vegetable Research and Development Centre, PO Box 42, Shanhua, Tainan, Taiwan, 74199, Department of Microbiology, The University of Queensland, Brisbane, Queensland 4072, Australia,* [5]*Department of Genetics and Developmental Biology, Monash University, Clayton, Victoria, 3168.*

(Received 12 December 1996 / Accepted 26 March 1997)

Abstract. Six *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*) genomic DNA fragments were isolated, using RAPD techniques and cloning, from the three genetically diverse strains: ACH092 (Biovar 4), ACH0158 (Biovar 2) and ACH0171 (Biovar 3) (1). One of these cloned fragments was selected because it was present constantly in all bacterial strains analysed. The remaining five clones were selected because Southern hybridisation revealed that each showed partial or complete specificity towards the strain of origin. A seventh genomic fragment showing a strain-specific distribution in Southern hybridisations was obtained by differential restriction, hybridisation and cloning of genomic DNA. Each of these clones was sequenced and primers to amplify the insert were designed. When DNA from the strain of origin was used as template, PCR amplification for each of these fragments yielded a single band on gel analysis. One pair of primers amplified the species-constant fragment of 281 bp from DNA of all *B. solanacearum* strains investigated, from DNA of the closely related bacterium which causes "blood disease" of banana (BDB) and in *P. syzigii.* The sensitivity of detection of *B. solanacearum* using these ubiquitous primers was between 1.3 and 20 bacterial cells. The feasibility and reliability of a PCR approach to detection and identification of *B. solanacearum* was tested in diverse strains of the bacterium in several countries and laboratories.

INTRODUCTION

Bacterial Wilt, caused by *Burkholderia solanacearum* E F Smith (previously named *Pseudomonas solanacearum* (3,4), is one of the most important plant diseases of bacterial origin in the world[2]. Most genera within the *Solanaceae* may be infected together with species from approximately 50 other families of higher plants (1,2). The disease is particularly destructive on crop plants in the tropics where tomato, groundnut, potato, banana, eggplant and ginger suffer serious reductions in both yield and quality. The disease is also important in many temperate regions where it causes economically significant damage particularly to potato and tomato crops. In some Asian countries *B. solanacearum* may be responsible for an overall 75% reduction in the potato crop (5).

Bacterial wilt disease is known to spread within and between countries in infected and latently infected seed potatoes (6), ginger (7) and banana corms and *Heliconia* rhizomes (8). In addition the disease may be spread *via* true seeds (7), seedlings (9), through mechanical damage during pruning and harvesting etc., or through roots damaged by nematodes (10). The bacterium may pass between apparently healthy roots (11) and

*Corresponding authour. Mailing address: Department of Genetics University of Adelaide, South Australia 5005, Australia. Telephone no.: Int+ 61 8 3034661. Fax no. : Int+ 61 8 303 4399. E-mail jtimmis@genetics.adelaide.edu.au.

Material may be protected by copyright law (Title 17, U.S. Code)

aerial transmission is possible through rain-splash (12). The bacterium is able to survive for long periods in some soil types and climates, though viable cells disappear rapidly in some other environments. The wide host range enables the organism to persist within weed species and it is often present in soils immediately after native vegetation has been cleared for crop production. Technically simple and reliable methods are therefore urgently needed for the identification of actual and potential sources of infection, for monitoring quarantine control of outbreaks of the disease and for assessing latent infections in plant material.

Attempts, either to breed resistant varieties or to apply soil and crop management regimes, have not been successful. Likewise quarantine and hygiene measures designed to control the pathogen have been unable to substantially reduce the spread of the disease in most countries. However, there is evidence for location-specific resistance in tomato[13] and other crops suggesting that it may be possible to breed resistant cultivars if the factors affecting infection and spread of the pathogen can be identified and incorporated into new varieties. To allow a greater understanding of the interaction between bacterial strains and host resistance sources, it is first necessary to elucidate the genetic diversity as it pertains to geographical distribution, host range and virulence. It is also essential to elucidate the mechanisms responsible for the generation of genetic diversity. None of the available classification systems adequately reflect the genotypic complexity of *B. solanacearum* (14), nor has the diversity in host range and variation in virulence been linked with readily determined, measurable characters.

It is apparent that appropriate subdivision of *B. solanacearum* is required before significant advances in disease control can be achieved and several advances towards this end have been made. Groupings based on RFLP analyses (2,15), gene sequencing (16) and PCR analysis of specific DNA sequences (15,17) have been developed. All of these approaches suffer from the problem that they are technically demanding and consequently unlikely to be adopted by laboratories in developing countries where research is required. We are seeking a reliable classification system which utilises minimal technical, financial and infrastructural resources and we now report our progress and prospects.

In this paper we report that one set of PCR primers to "strain constant" DNA facilitates the specific detection of all strains of *B. solanacearum* whilst a range of primer pairs designed to amplify "strain variable" DNA is demonstrated to show potential as the basis of a powerful approach to strain differentiation and identification.

MATERIALS AND METHODS

Bacterial strains. Three strains of *B. solanacearum* were chosen as representatives of a wide range of host specificity (1). These strains were isolated in Queensland, Australia as ACH092 (Biovar 4, from ginger), ACH0158 (Biovar 2, from potato) and ACH0171 (Biovar 3, from eggplant). SA1S and SA1R are Biovar 2 strains isolated from potato in South Australia. Other strains of *B. solanacearum* and other bacterial species and strains are described in the text or in figure legends.

Pathogenicity assay. As a minimum test of strain integrity, *B. solanacearum* strains were tested for the ability to induce a hypersensitive response (HR) on tobacco (*Nicotiana tabaccum*) plants by leaf tissue infiltration. Cultures were grown in tetrazolium broth to OD_{600} = 1.0-1.5. Approximately 0.25 ml of culture were infiltrated under the upper surface of the leaf and plants were placed in a growth cabinet (8 hour day, 30°C; 16 hour night, 25°C) until examination for the HR after 16 h.

METHODS

Restriction enzyme digests. Restriction enzyme digests were carried out according to the conditions recommended by the manufacturers (Boehringer-Mannheim) using 10 units (U) of enzyme per µg of DNA. In addition, spermidine was added to a final concentration of 3 mM. Ribonuclease A was added to a final concentration of 20 µg/ml in plasmid DNA digests and incubation was for at least 3 h at the appropriate temperature.

Agarose gel electrophoresis. DNA was separated by electrophoresis in 0.8 to 1.5% agarose gels (18). On completion of electrophoresis, the DNA was stained with ethidium bromide and viewed under UV light. DNA fragment size was determined using a computing program based on that of Duggleby *et al.* (19).

Pulsed-field gels were performed as described[20] except that chloramphenicol was used to align chromosomal replication origins. *Spe*I and marker DNA fragments were resolved in 1.2% agarose (Promega) gels for 40 hr with a pulse time of 15 sec. Lambda DNA multimers for size markers were prepared as directed by the manufacturer (NEB). Gels were blotted onto Hybond™-N membrane filters (Amersham) and hybridised with oligo-labelled λ DNA (size markers) or the oligo-labelled 281 bp fragment (*SpeI* fragments of DNA from *B. solanacearum* strains).

Recovery of DNA from agarose gels and cloning. DNA was recovered from agarose gels using the freeze-squeeze method described by Thuring *et al*[21].

Material may be protected by copyright law (Title 17, U.S. Code)

Fragments were ligated into the TA cloning site of the vector pCR™ II (Invitrogen). Recombinant molecules were transformed into *E. coli* host strain INVaF'. The *Eco*RI restriction sites flanking the TA cloning site of the vector allowed the cloned inserts to be isolated.

Preparation of genomic DNA. DNA was prepared from *Pseudomonas* and *Burkholderia* spp. by the method of Chen and Kuo (22). Bacterial species were cultured in nutrient broth except for *B. solanacearum* which was cultured in tetrazolium broth. A bacterial cell pellet was obtained from 1.5 ml of culture by centrifugation at 13000 x g for 3 min. *B. cepacia* cells were obtained by scraping colonies from plates. Cells were lysed by the addition of lysis buffer (22) followed by vortexing. Protein and cell debris were removed by increasing the salt concentration to 0.5 M NaCl followed by centrifugation at 13000 x g for 10 min. Ribonuclease A was added to the supernatant to a final concentration of 10 μg/ml and incubated at 37°C for 30 min.. After chloroform deproteinisation, the aqueous phase was ethanol precipitated and the DNA resuspended in 1 x TE.

Purification of plasmid DNA. Prior to phenol / chloroform extraction, RNase A was added to small-scale plasmid preparations to a final concentration of 40 μg/ml and samples incubated for 20 min at 37°C. This solution was deproteinised twice with phenol / chloroform and ethanol precipitated. The resulting DNA pellet resuspended in distilled H_2O and precipitated with PEG_{8000} (18). The precipitate was washed with cold 70% ethanol and resuspended in H_2O. For some experiments plasmid DNA was further purified on CsCl gradients (18).

Probe preparation Southern blotting, hybridisation and autoradiography. Plasmid inserts to be radioactively labelled were isolated from an agarose gel and labelled using the oligolabelling method (23). Labelling was accomplished using a GIGAprime™ DNA labelling kit (Bresatec, South Australia) and $\alpha[^{32}P]$-dATP (Bresatec). Unincorporated nucleotides were removed by centrifugation through Bio-Gel. The specific activity of the probe was determined by scintillation counting. Colony hybridisations were as described by Grunstein and Hogness (24).

DNA was transferred (25) from agarose gels to Hybond™-N^+ membranes (Amersham) by the method described by the manufacturers (Amersham: protocols for nucleic acid blotting and hybridization). Prehybridization buffer was added to nitrocellulose membranes which were incubated at 65°C for at least 2 h. The probe, plus salmon sperm DNA, was denatured in alkali and then neutralised. Hybridisation was at 65°C for at least 16 h. Membranes were subsequently washed at 65°C firstly in a solution of 2 x SSC, 0.1% SDS for 30 min followed by 1 x SSC, 0.1% SDS and 0.5 x SSC, 0.1% SDS for 30 min each. Membranes were wrapped in plastic film to prevent drying and exposed to X-ray film (Fuji) in a cassette with an intensifying screen (unless otherwise stated) for a periods varying between 1 hour at room temperature to 7 days at -80°C.

DNA Sequencing and Sequence Analysis. *B. solanacearum* DNA cloned into the vector pCR™ II was sequenced either by automated sequencing or manually by the dideoxy chain termination method (18). Sequence comparisons were performed by a homology search of the GenBank data base using the "Fasta" algorithm (26) of the Genetics Computer Group Sequence Analysis Software Package Version 7.0 (27).

Designing oligonucleotide primers. RAPD primers were obtained from Operon Technologies Inc., USA. Other PCR primers were synthesised on a Beckman oligo synthesiser after checking for absence of homology between primers. The secondary structure of each primer was examined using the "squiggles" program of the Genetics Computer Group Sequence Analysis Software Package Version 7.0 (27).

PCR amplifications. PCR amplifications were performed at various Australian and Asian centres using various brands of programmable temperature controllers (ARN, South Australia; Corbett Research). Initially PCR reactions were conducted in a 25 or 50 μl volume containing 1 x PCR buffer (Sambrook *et al.*, 1989), 5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.5 pmol/μl of primers, 0.2 ng/ml of genomic template DNA and 0.055 U/μl of Taq DNA polymerase. Each reaction was overlayed with mineral oil (Sigma) and heated to 95°C for 2 min to denature the template DNA. Samples were then cycled 30 times with each cycle consisting of a 30 sec. denaturation step at 94°C (except for 771/772 which required 1 minute), a 30 sec annealing step and a 1 minute extension step at 72°C. Pairs of primers required different annealing temperatures. These were: 759/760: 53°C, 761/762: 53°C, 763/764:63°C, 765/766: 54°C, 767/768: 68°C, 769/770: 53°C and 771/772: 60°C. A 5 μl aliquot of PCR product was electrophoresed in 1.5% agarose gels.

Optimisation of the PCR reaction for primers 759 / 760 identified 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 0.5 pmol/μl of primers, 1 ng/μl of genomic template DNA and 0.01 U/μl of *Taq* DNA polymerase. Samples were denatured at 94°C for 3 min, annealed at 53°C for 1 min and extended at 72°C for 1.5 min, followed by 30 cycles of 94°C for 15 sec., 60°C for 15 sec., 72°C for 15 sec. and a final extension of 72°C for 5 min.

Material may be protected by copyright law (Title 17, U.S. Code)

RESULTS

Generation of strain-specific and strain-common DNA probes.

From differential random amplified polymorphic DNAs (RAPDs). A range of RAPD primers was applied to DNA from strains ACH0158, ACH0171 and ACH092 and the PCR products were resolved on agarose gels (Fig. 1). For each DNA sample, individual primers gave characteristic sets of PCR products which varied between primers in containing zero to many fragments of different molecular size and staining intensity (Fig. 1). Comparisons between strains suggested that some common bands were amplified by individual primers but for each primer type there was generally a large amount of heterogeneity of the amplified fragments between strains. Reproducibility of the RAPD banding patterns was found to be good when highly purified DNA samples were used as template (Fig. 1 cf lanes 1 -3 and 4 - 6). However, considerable variation was observed between replicates when more crudely prepared DNA samples were used as template for the PCR reaction (result not shown). It was consequently concluded that direct RAPD analysis was unsuitable as a reproducible approach to species and strain identification.

The large amount of genomic variation revealed by RAPD analyses indicated that many portions of the *B. solanacearum* genome were variable between strains and suggested these regions as potential probes for use in species and strain identification. There are two possible explanations for the variation revealed by RAPD PCR: firstly that it was due to minor nucleotide sequence differences in the DNA binding the primers or secondly, that the DNA targeted by the primers is present in some strains but absent from others.

To test which of these explanations is correct, several PCR fragments which appeared to be either common to all three strains of the organism or unique to a particular strain was excised from gels and eluted from the agarose. In most cases bands were chosen because they appeared to be unique RAPD identifiers of particular *B. solanacearum* strains. These fragments were labelled with ^{32}P and used individually as probes to Southern blots of *Eco*RI-digested genomic DNA from the three experimental strains and to DNA from two additional South Australian biovar 2 strains (SA1S and SA1R) which were isolated from potato (Fig. 2). In the majority of cases hybridisation identified DNA fragments from the strain of origin but no hybridisation to the other experimental strains (Fig. 2). Lower stringency hybridisation and washing than that illustrated gave the same results suggesting that minor sequence variation between strains affecting primer binding was not the source of the RAPD banding differences. Rather it appears that a proportion of sequences are present in some *B. solanacearum* genomes but are absent in others. It also appears that such sequences are by no means rare in occurrence because their presence was readily revealed using a small number of random PCR primers and isolated fragments. The three strains used in these experiments clearly contain a range of sequences whose presence or absence may uniquely characterise each bacterial genome. In all cases SA1S and SA1R behaved similarly to ACH0158, suggesting that biovar 2 has some degree of genomic similarity and homogeneity.

In contrast to these variable probes, one eluted RAPD fragment, in this case chosen for its commonality between strains, hybridised to an invariable 1.3 kb band in Southern hybridisations to *Eco RI*-digested genomic DNA (Fig. 2f). This class of fragment was initially selected in order to provide positive control fragments in Southern hybridisations and PCR analyses.

From strain-specific genomic restriction fragments. Gel resolution of *Pst*I-digested ACH0158 DNA showed four bands of approximately 14.6, 18.6, 21.6 and 27.6 kb which were absent from a comparable digest of the ACH0171 genome (result not shown). On Southern analysis, these bands did not hybridise with a total ACH0171 genomic probe (result not shown). The three largest fragments were eluted together from the gel, further restricted with *Sau*3A and cloned at the *Bam*HI site of pUC19 or into pUC19 digested with both *Bam*HI and *Pst*I. Several clones were recovered of which one (pBs2.10) hybridised to the 27.6 kb *Pst*I band and to an *Eco*RI band of approximately 5.0 kb in ACH0158 DNA but showed no hybridisation to DNA from strains ACH092 or ACH0171 (Fig. 2b). The probe which was specific for ACH0158 (biovar 2) also hybridised to fragments of the same mobility in SA1S and SA1R (Fig. 2b). Other clones derived in this way hybridised to polymorphic bands in both ACH0158 and ACH0171 DNA (result not shown).

Development of single site-directed PCR primers. The 570 bp insert of pBs2.10 was sequenced and the information used to design (Table 1) forward (630) and reverse (631) primers to amplify a 307 bp region of the genome by PCR. Application of these primers to ACH0158 DNA produced the predicted single fragment and the lack of products amplified from ACH092 and ACH0171 DNA confirmed that the sequence is absent from these two strains (result not shown).

Seven additional apparently informative fragments (6 strain-variable and 1 strain-constant) were identified by RAPD PCR and Southern analyses as described above. These PCR fragments

Material may be protected by copyright law (Title 17, U.S. Code)

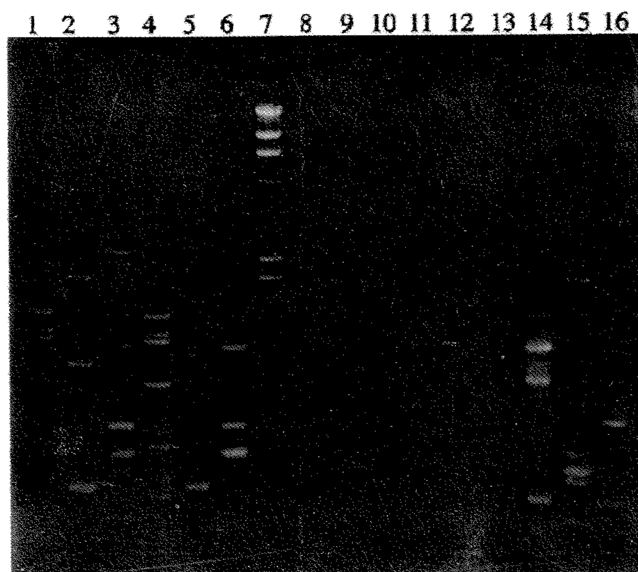

Figure 1. Examples of random amplification of polymorphic DNA (RAPD) from *B. solanacearum* strains 092, 0158 and 0171. Genomic DNAs from strain ACH0171 (lanes 1, 4, 8, 11, 14); ACH0158 (lanes 2, 5, 9, 12, 15) and ACH092 (lanes 3, 6, 10, 13, 16) were amplified with RAPD primers from kit D (Operon, USA). Lanes 1 - 6, primer 18; lanes 8 - 10, primer 17; lanes 11 - 13, primer 19; lanes 14 - 16, primer 20. Amplified fragments were resolved on 1.4% agarose gels, stained with ethidium bromide and photographed under light at 300 nm. Lane 7 contains λ bacteriophage DNA digested with Hind III to give fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. Figure produced by Adobe Photoshop™.

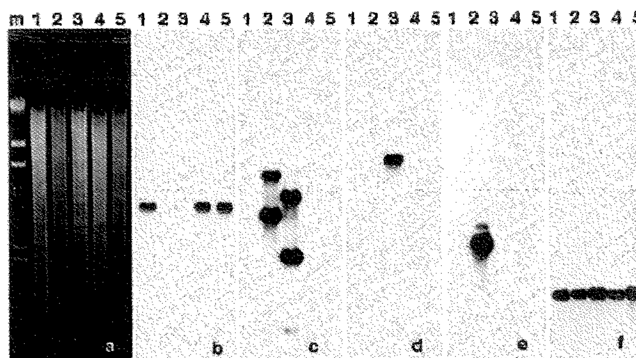

Figure 2. Southern hybridisations using specific probes to *B. solanacearum* DNA. Samples of DNA from *B. solanacearum* strains ACH0158, ACH092, ACH0171, SA1S and SA1R (lanes 1 - 5 respectively in a - f) were restricted with *Eco* RI and the fragments resolved on 0.8% agarose gels. (a) The gel was stained in ethidium bromide and photographed under light at 300 nm. The first lane (m) contains λ bacteriophage DNA digested with Hind III to give fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. Fragments of DNA from similar gels were transferred to nylon membrane and probed with: (b) the labelled insert derived from an ACH0158-specific cloned fragment (pBs2.10), (c) a labelled fragment excised from a gel resolution of PCR-amplified fragments derived from strain ACH0171 DNA primed with RAPD D7, (d) a labelled fragment excised from a gel resolution of PCR-amplified fragments derived from strain ACH0171 DNA primed with RAPD D10, (e) a labelled fragment excised from a gel resolution of PCR-amplified fragments derived from strain ACH092 DNA primed with RAPD D3, (f) a second labelled fragment excised from the same gel as the fragment in (e). Figure produced by Adobe Photoshop™.

Material may be protected by copyright law (Title 17, U.S. Code)

Table 1. Extended primers for strain differentiation in *B. solanacearum*.

RAPD primers used to amplify the fragment internal to the primers is underlined in each case. Sequences are shown 5' to 3'. * indicates forward (f) or reverse (r) primers.

Primer	Clone	Sequence
630f*	pBs2.10	ATACAGAATTCGACCGGCAC
631r*		AATCACATGCAATTCGCCTAC
759f	092-03B5	GTCGCCGTCAACTCACTTTCC
760r		GTCGCCGTCAGCAATGCGGAATCG
761f	092-03A #8	GTCGCCGTCACCCAACCTTTCCTCG
762r		GTCGCCGTCAAACTCGATCACTCTG
763f	092-03A #18	GTCGCCGTCACGCAATCTCATCCAC
764r		GTCGCCGTCAGCAAGGATGTC
765f	0158-19 #13	CTGGGGACTTCCTTGGTGTG
766r		CTGGGGACTTACCGTGGCCAAGGC
767f	0171-10 #9	TTGGCACGGGCGCGAACGGCAAG
768r		TTGGCACGGGTAATGAGCAGATCGG
769f	0171-03 #4	GTCGCCGTCACCTTCCCAG
770r		GTCGCCGTCATGCTCGCCCAACGAC
771f	0171-7A #11	TTGGCACGGGCGCGAACGGCAAGTC
772r		TTGGCACGGGTGATGAGCAGATCGG

were cloned and sequenced in both directions (data not shown). These data were used to search the GenBank data base using the *Fasta* algorithm (26) but none showed significant similarity with any previously characterised nucleic acid sequences. As expected, each of the cloned inserts was flanked by 10 nucleotides characteristic of the particular RAPD primer used in their amplification. Using these 10 nucleotides and a further 10-16 nucleotides 3' to the RAPD primer, extended nucleotide primers were designed to confine amplification to the specific cloned fragment (Table 1). The variable primer lengths were necessary to avoid cross-homology, to avoid problems of intrastrand

Material may be protected by copyright law (Title 17, U.S. Code)

secondary structure and to maintain a constant GC composition.

These modified primers were used to amplify genomic DNA from strains ACH092, ACH0158 and ACH0171 (Fig. 3). Most amplifications yielded a single band (Fig. 3, lanes 3, 6, 10, 14, 18 and 20) identical in size to the corresponding RAPD-amplified band from which they were derived. As predicted from the Southern analyses (Fig. 2), these unique bands were present only after PCR amplification of the "parent" DNA. The additional nucleotides added to the RAPD primer therefore reduce binding to single site for each primer and leads to the amplification of a unique strain-specific genomic segment provided the latter is present in the genomic DNA sample. In a minority of cases (Fig. 3, lanes 9 and 15) more than one band may be seen and strain-specificity is not absolute, but in such cases the most abundant PCR product still corresponds to the "parental" genomic fragment. Primers 771/772 amplify a similar sized band from ACH092 and ACH0171 which is absent from ACH0158 DNA. This suggests that ACH092 and ACH0171 are more closely related to each other than to ACH0158.

Primers 759/760, which define the fragment which hybridises to a common 1.3 *Eco*RI band in Southern hybridisations (Fig. 2f), gave rise to a constant 281 bp amplification product in all *B. solanacearum* strains tested (Fig. 4). It was concluded that this small portion of the genome is a component of a highly conserved region essential to the function of all, or at least a broad spectrum of, *B. solanacearum* strains.

Wider tests of the ability of the PCR primers to distinguish groups of *B. solanacearum* genotypes. The above results suggested that the genomes of the three experimental *B. solanacearum* strains contain both variable and constant DNA which may be utilised for species and strain identification and detection.

Species specificity and detection of the pathogen. Primers 759/760 were used to amplify DNA from a wide range of Australian, Philippines and Taiwanese *B. solanacearum* isolates (Fig. 4, 5). A constant 281 bp fragment was amplified from all isolates of *B. solanacearum* and also from Indonesian isolates of *P. syzygii* (the causative agent of Sumatra disease of clove) and "*P. celebensis*" - the bacterium causing banana Blood Disease. Experiments in Japan (Shin-ichi Ito, personal communication) confirm these results. In contrast the primers gave no products to many other soil and plant-pathogenic bacterial DNA templates (eg Fig. 4b). Species tested included *B. pickettii* (previously *Pseudomonas picketii*), *Pseudomonas andropogonis, P. aeruginosa, P. corrugata, Burkholderia cepacia, P. putida and P. fluorescens* (Fig. 4b). Genomic DNA of other species which show no hybridisation to this fragment in Southern analyses include 6 races of *Xanthomonas campestris* pv. *vesicatoria*, 2 strains

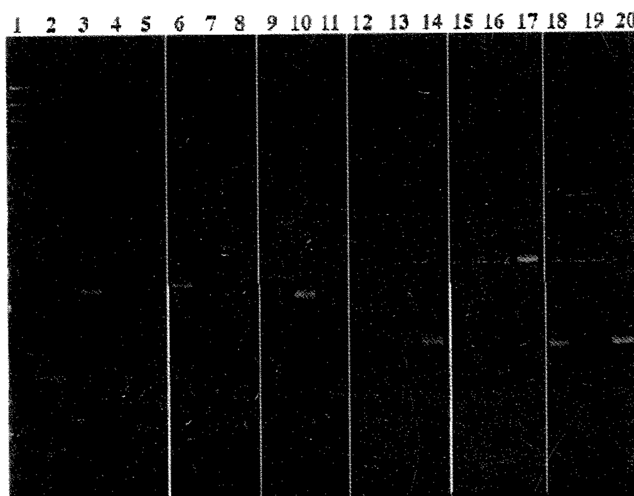

Figure 3. Samples of DNA from *B. solanacearum* strains amplified with primers which yield differentially occurring PCR products. Lanes 3 -5: primers 761/762 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively; lanes 6 -8: primers 763/764 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively; lanes 9 -11: primers 765/766 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively; lanes 12 -14: primers 767/768 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively; lanes 15 -17: primers 769/770 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively; lanes 18 - 20: primers 771/772 used to amplify DNA samples from *B. solanacearum* strains ACH092, ACH0158 and ACH0171 respectively. Lane 1 contains λ bacteriophage DNA digested with Hind III to give fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb. Lane 2 contains pUC19 DNA digested with HpaII to give fragments of 501, 489, 404, 331, 242, 190, 147 bp. Figure produced by Adobe Photoshop™.

Material may be protected by copyright law (Title 17, U.S. Code)

of *Xanthomonas campestris* pv. *campestris*, 2 strains of *Xanthomonas campestris* pv. *glycenia*, *Erwinia chrysanthemi*, *B. cepacia* and *B. gladioli* (results not shown). These results indicate that the 281 bp fragment specifically identifies the genome of *B. solanacearum* and that it may be useful tool in disease diagnosis, in monitoring the extent of contamination in seeds, vegetative propagules and soil and also in establishing the rate of spread of the bacterium in different genotypes.

The sensitivity of the PCR assay in detection of B. solanacearum DNA. Figure 5b shows PCR analyses of a dilution series of *B. solanacearum* DNA covering the range from 25 ng to 2.5 pg of template DNA. The 281 bp fragment could be detected by PCR in dilutions equating to approximately 1.4 - 20 cells in different assays (Fig. 5b, lane 7).

The location of the 281 bp common fragment on the B. solanacearum genome. Genomic DNA from strains AW1, GM1000 and PS1000 (=ACH0171) (28) was restricted with *Spe*I and the resulting large fragments resolved by pulsed-field gel electrophoresis (Fig. 6a). Southern hybridisation of these fragments using the 281 bp fragment as probe (Fig. 6b) indicates that it is present, probably as a single copy, on 190 kb *Spe*I fragments of both the GMI1000 and ACH0171 genome (Fig. 6b, lanes 3 and 4 respectively). In contrast, strain AW1 DNA contains at least 2 copies of this sequence on *Spe*I fragments of 260 and 380 kb (Fig. 6b, lane 2).

DISCUSSION

The results described in this paper show that there are likely to be many portions of the *B. solanacearum* genome which vary in a quantitative manner, being present in some genomes and absent from others. The variation described appears not to be nucleotide sequence variation such as that previously, now routinely, used to obtain group-specific or group-variable primers (for example, that present in ribosomal DNA and pathogenicity genes). Rather, it involves the presence or absence of what must be substantial portions of the genome and the detection and use of these regions is a novel approach to prokaryotic genome analysis.

There is much scope for the utilisation of genomic variation in the identification of genetic diversity and some progress has been made in relating DNA sequence data to biological characteristics (5,19,30,31). Such studies are particularly important to provide a clear understanding of the epidemiology, biological diversity and virulence of plant pathogens. However, molecular approaches that are biologically subtle and informative, as well as technically simple, are not yet available for the study of populations of phytopathogens and their interactions with host plants. Amplification by PCR has been successfully used in surveillance of outbreaks and emergence of new strains of *Vibrio cholerae* (32), in quality control in *Rhizobium* inoculants (33), in detection of methanotrophic bacteria in the environment (34) and in the identification and phylogenetic analysis of cyanobacteria contributing to algal blooms (35). Approaches using PCR to detect commonality and specificity in genomes normally make use of sequence mismatch within the primers as the basis of resolution. This specific recognition requires subtle control of buffer conditions and primer annealing temperature. For example a 2°C lowering of the annealing temperature may abolish PCR specificity where this is reliant on only 1 or 2 mismatched base pairs out of 25 primer nucleotides. This is the case where genes commonly used in phylogenetic analyses, such as 16S rRNA genes which share close homology in all prokaryotes, are targeted (17). These problems cannot occur when the template sequences themselves are either present or entirely absent, thus potentially allowing a more robust experimental procedure without the necessity for fine control of buffer conditions and annealing temperatures. This is important in *B. solanacearum* as analyses are required in developing countries where limited technical and infrastructural resources are available. A more extensive screen of *B. solanacearum* strains in Asia will determine whether subdivisions of the species have biological importance and utility for plant breeders and pathologists.

The results obtained here compare and contrast with the results of Seal *et al.* (17) who used subtractive hybridisation to obtain *B. solanacearum*-specific probes and primers. A subtracted library was made using a single *B. solanacearum* strain (UW25) as tracer DNA and *Xanthomonas campestris* pv. vesicatoria was used as driver. A single clone of 44 tested in these experiments hybridised to a majority (82 out of 85) of *B. solanacearum* strains tested and PCR primers designed to amplify the sequence showed similar specificity. These primers also amplified a larger fragment from *P. syringae* pv. tabaci and *B. pickettii*. The use of longer amplified template DNA of all 85 *P. solanacearum* strains and there was no product from DNA of *P. syringae* pv. tabaci or *B. peckettii*. Similar sensitivities of detection to those reported here were achieved and amplification was also demonstrated in the presence of large excesses of non-template DNA and in total DNA from infected plant tissue (17).

Material may be protected by copyright law (Title 17, U.S. Code)

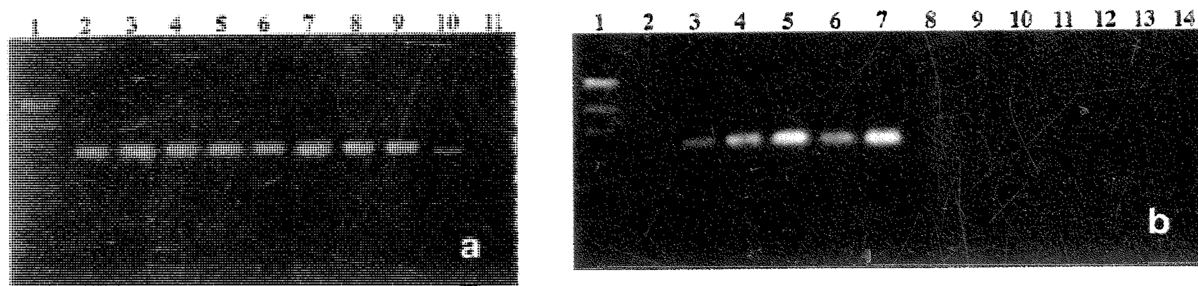

Figure 4. Samples of DNA from *B. solanacearum* strains amplified with primers which yield constantly occurring PCR products. (a) Primers 759/760, designed to amplify a 281 bp product containing the sequence used as probe in Fig. 2f were used in PCR reactions on template DNA from *B. solanacearum* strains 006, 012B. 016, 0190, 01062, 01068S, 01082, 01085D (lanes 2 - 9 respectively) and the positive control ACH092 (lane 10). As a negative control, no DNA was included in the reaction leading to lane 11. Lane 1 shows pUC19 DNA digested with HpaII to give fragments of 501, 489, 404, 331, 242, 190, 147 bp. (b) The same primers applied to template DNA from *B. solanacearum* strains ACH092, ACH0158, ACH0171, SA1S and SA1R (lanes 3 - 7 respectively) and DNA samples from *P.aeruginosa*, *P. corrugata* 104, *P. corrugata* 117, *B. cepacia*, *P. fluorescens* 74, *P. fluorescens* 96 and *P. putida* (lanes 8 - 14 respectively). As a negative control, no DNA was included in the reaction leading to lane 2. Lane 1 shows pUC19 DNA digested with HpaII to give fragments of 501, 489, 404, 331, 242, 190, 147 bp. Figure produced by Adobe Photoshop™.

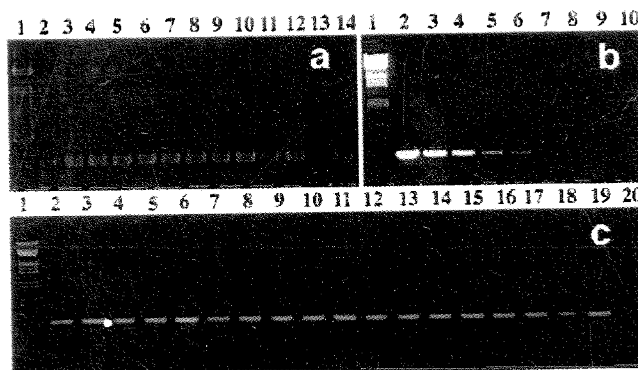

Figure 5. The ubiquity and sensitivity of detection of the 281 bp sequence flanked by primers 759/760. (a) PCR products to templates of Taiwanese and control strains of *B. solanacearum*. Primers 759/760 were used to amplify template DNA from ACH092, ACH0171, Pss4, Pss5, Pss11, Pss27, Pss37, Pss43, Pss60, Pss70, Pss71, Pss105 and Pss107 (lanes 2 - 14 respectively). A 100 bp ladder is included as molecular size markers in lane 1. (b) Serial dilution of *B. solanacearum* strain CIP10 DNA. PCR products to 25 ng of template DNA using primers 759/760 (lane 2) and zero DNA (lane 10). Lane 3 contains DNA from 137,500 viable cells followed by serial 10-fold dilutions (lanes 4 - 9). Lane 1 contains λ bacteriophage DNA digested with HindIII + *Eco* RI to give fragments of 23.1, 21.3, 9.4, 6.6, 5.1, 4.9, 4.4, 4.2, 3.5, 2.3, 2.0 and 1.6 kb. (c) PCR products to templates of an international collection of *B. solanacearum* and control strains. Primers 759/760 were used to amplify template DNA from CIP296, CIP310, 039, 780, 792, CIP243, 290,314, 205, 809, 222, 012, 1071, CIP369, CIP365-93, Br1, 291, 288 (lanes 2 - 19 respectively). Lane 1 contains λ bacteriophage DNA digested with HindIII+ *Eco* RI to give fragments of 23.1, 21.3, 9.4, 6.6, 5.1, 4.9, 4.4, 4.2, 3.5, 2.3, 2.0 and 1.6 kb. As a negative control, template DNA was not included in the reaction for lane 20. Figure produced by Adobe Photoshop™.

Material may be protected by copyright law (Title 17, U.S. Code)

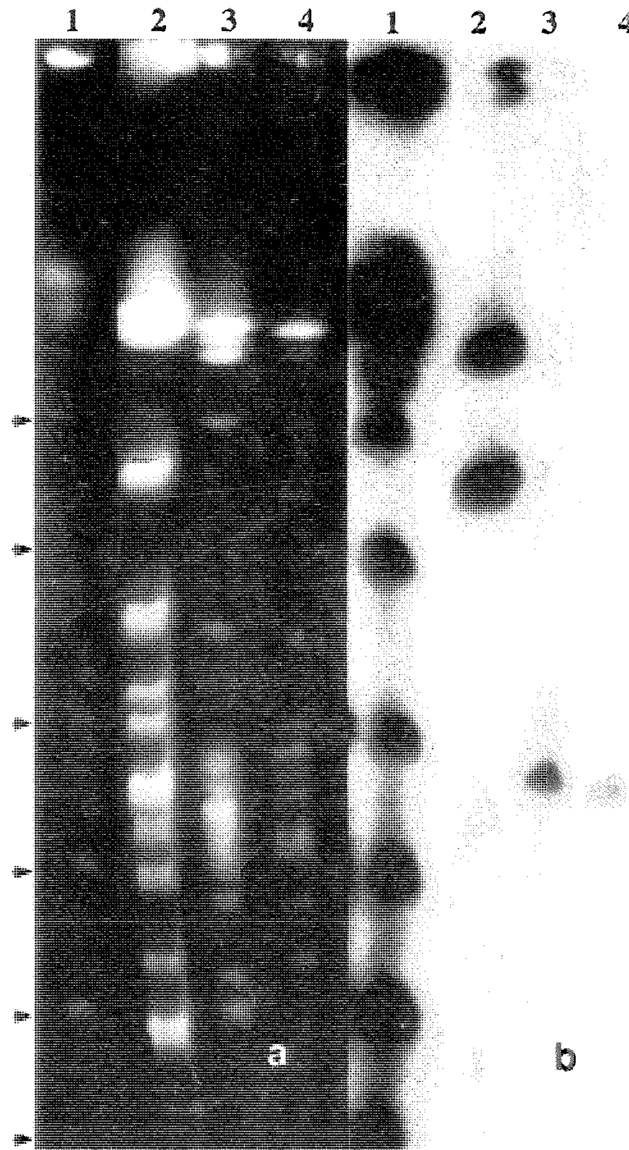

Figure 6. Southern hybridisation of the 281 bp fragment to *Spe I*-digested DNA from three strains of *B. solanacearum*. (a) Lane 1: Ethidium bromide-stained fragments of λ multimer molecular size standards. Arrows indicate marker fragments of 291.0, 242.5, 194.0, 145.0, 97.0 and 48.5 kb. Lanes 2 -4: Ethidium bromide-stained *Spe I* fragments of *B. solanacearum* strain AW1, GMI1000 and PS1000 (=ACH0171). (b) Autoradiograph of a Southern transfer of (a). Lanes 2-4 were hybridised with the ^{32}P labelled 281 bp fragment. Lane 1, containing λ markers, was hybridised separately with ^{32}P labelled λ DNA. Figure produced by Adobe Photoshop™.

It is surprising that the subtractive cloning approach (17) did not yield more clones of the type of DNA which is variable between strains. A single *B. solanacearum* strain was used to derive the 44 subtracted clones analysed by Seal *et al.* (17). Of these 15 were not of *B. solanacearum* DNA and 12 contained DNA which was repeated to various extents in the AW25 genome and which varied extensively in copy number within other strains tested. It is not clear whether any of these were entirely absent from some genomes in the same way as our strain-variable DNA and the nature of the

Material may be protected by copyright law (Title 17, U.S. Code)

remaining clones is not reported. Our strain-variable DNA does not appear to be repeated. Single copy status has been established for the 281 bp fragment in strains GMI1000 and ACH0171 although there are at least 2 copies of this sequence in AW1 DNA. It appears that subtractive cloning yielded quite a different result from our experimental approach and the paucity of strain-variable DNA in the former is inconsistent with its predominance and ready identification in our experiments.

Our experiments suggest large-scale variation in presence/absence of specific sequences within the *B. solanacearum* gene pool. Some of this variation may be due to presence or absence of plasmids, but this explanation seems unlikely to be adequate in itself. It is possible that the main chromosome(s) of different ecotypes and strains varies considerably in size and in gene content. Superimposed on this is the evidence from Southern hybridisation of pulse-field gels (Krishnapillai, unpublished results) that specific sequences may reside on the main chromosome in some strains and on a megaplasmid in others. Lawrence and Roth (16) showed that, because they did not hybridise with the corresponding genes of other enteric bacteria, the cobalbumen synthetic genes and the genes providing cobalbumen-dependent diol dehydratase have been introduced into the Salmonella lineage by horizontal transfer from an exogenous source. On this basis, genomic flux by periodic loss and reacquisition of genes has been suggested to be an important aspect of bacterial population genetics and evolution[36]. The tracts of DNA which distinguish the genotypes in *B. solanacearum* may come within a similar category of DNA which is lost or gained in response to selection in particular plant and soil environments.

Acknowledgments. We are grateful to the Australian Centre for International Agricultural Research and the Australian Research Council for financial support.

REFERENCES

1. Hayward, A. C. (1994). The hosts of *Pseudomonas solanacearum*. In: Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum*. Ed. Hayward and Hartman. CAB International, UK. pp 9-24.
2. Kelman, A., Hartman, G. L., Hayward, A. C., (1994). Introduction. In: Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum*. Ed. Hayward and Hartman. CAB International, UK. pp 1-8.
3. Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes) comb. nov. Microbiol. and Immunol. 36: 1251-1275.
4. Anonymous. (1993). Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List number 45. Int. J. System. Bacteriol. 43: 398-399.
5. Cook, D., Sequeira, L. (1994). Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. In: Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum*. Ed. Hayward and Hartman. CAB International, UK. pp 77-93.
6. Ciampi, L, Sequeira, L. (1980). Multiplication of *Pseudomonas solanacearum* in resistant potato plants and the establishment of latent infections. American Potato J. 57: 319-329.
7. Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *P. solanacearum*. Ann. Rev. Phytopathol. 29: 65-87.
8. Akiew, E. B., Hyde, K. Diatloff, L., Peterson, R. (1990). Bacterial wilt of *Heliconia* plants from Oahu, Hawwaii. ACIAR Bacterial Wilt Newslett. 6: 5.
9. Hsu, S. T. (1991). Ecology and control of *Pseudomonas solanacearum* in Taiwan. Plant Protection Bull., Taiwan, 33: 72-79.
10. Napiere, C. M. (1980). Varying inoculum levels of bacteria - nematodes and the severity of tomato bacterial wilt. Ann. Trop. Res. 2: 129-134.
11. Kelman, A., Sequeira, L. (1965). Root-to-root spread of *P. solanacearum*. Phytopathol. 55: 304-309.
12 Ono, K. (1983). Ecological studies on the bacterial wilt of tobacco caused by *Pseudomonas solanacearum* E. F. Smith. III Distribution and spread of the pathogen in infected tobacco field under rainfall. Bull. Okayama Exptl. Station 42: 149-153.
13. Hanson, P. M.,. Wang, J. - F., Licardo, O., Mah, S. Y., Hartman, G. L. , Lin, Y. - C., Chen, J.- T. (1995). Variable reaction of tomato lines to bacterial wilt evaluated at several locations in Southeast Asia. *Hort Science* 31, 143-146.
14. Hayward, A. C. (1964). Characteristics of *P. solanacearum*. J. Appl. Bacteriol. 27: 265-277.
15. Gillings, M., Fahy, P. Davies, C. (1993). Restriction analysis of an amplified polygalacturonase gene fragment differentiates strains of the phytopathogenic bacterium *P. solanacearum*. Lett. Appl. Microbiol. 17:

Material may be protected by copyright law (Title 17, U.S. Code)

44-48.

16. Lawrence, J. G., Roth, J. R. (1996). Evolution of coenzyme B_{12} synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. Genetics 142: 11-24.
17. Seal, S.E., Jackson, L. A., Daniels, M. J. (1992). Isolation of a *P. solanacearum*-specific DNA probe by subtractive hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the Polymerase Chain Reaction. Appl. Environ. Microbiol. 58: 3751-3758.
18. Sambrook, J., Fritsch E.F., Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* (2nd Ed.). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, USA.
19. Duggleby, R. G., Kinns, H., Rood, J. I. (1981). A computer program for determining the sizes of DNA restriction fragments. Analyt. Biochem. 110: 49-55.
20. Ratnaningsih, E., Dharmsthiti, S., Krishnapillai, V., Morgan, A., Sinclair, M., Holloway, B. W. (1990). A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. J. Gen. Micro. 136: 2351-2357.
21. Thuring, R. W. J., Sanders, J. P. M. Borst, P. (1975). A freeze squeeze method for recovering long DNA from agarose gels. Analyt. Biochem. 66: 213-220.
22. Chen, W.-P., Kuo, T.-T. (1993). A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. Nucleic Acids Res. 21: 2260.
23. Feinberg, A.P., Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analyt. Biochem. 132: 6-13.
24. Grunstein, M., Hogness, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl Acad Sci, USA. 72: 3961-3965.
25. Southern, E. M. (1975). Detection of Specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98: 503-517.
26. Pearson, W. R., Lipman, D. J. (1988). Improved tools for biological sequence comparisons. Proc. Natl. Acad. Sci. (USA). 85: 2444-2448.
27. Devereux, J., Haeberli, P., Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387-395.
28. Holloway, B. W., Krishnapillai, V., Escuadra, M. D. (1994). Whole-genome analysis of Pseudomonads and its application to *Pseudomonas solanacearum.* In: Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum.* Ed. Hayward and Hartman. CAB International, UK. pp 59-76.
29. Cook, D., Barlow, E., Sequeira, L. (1989). Genetic diversity of *Pseudomonas solanacearum:* Detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol. Plant-Microbe Int. 2: 113-121.
30. Smith J. J., Offord, L. C., Holderness, M., Saddler, G. S. (1995). The genetic diversity of *Burkholderia solanacearum (*Synonym *Pseudomonas solanacearum)* Race 3 in Kenya. *Applied and Environmental Microbiology* 61, 4263-4268.
31. Frey, P., Smith, J. J., Albar, L., Prior, P., Saddler, G. S., Trigalet-Demery, D., Trigalet, A. (1996). Bacteriocin typing of *Burkholderia (Pseudomonas) solanacearum* Race 1 of the French West Indies and correlation with genomic variation of the pathogen. *Applied and Environmental Microbiology* 62, 473-479.
32. Rivera, I. G., Chowdhury, M. A. R., Huo, A., Jacobs, D., Martins, M. T., Colwell, R. R. (1995). Enterobacterial repetitive intergenic consensus sequences and PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* 01,039, and Non-01 strains. Appl. Environ. Microbiol.. 61: 2898-2904.
33. Tas, E., Saano, A., Leinonen, P., Lindstrom, K. (1995). Identification of *Rhizobium* spp. in peat-based inoculants by DNA hybridisation and PCR and its application in inoculant quality control. Appl. Environ. Microbiol.. 61: 1822-1827.
34. McDonald, I. R., Kenna, E. M., Murrell. J. C. (1995). Detection of methanotrophic bacteria in environmental samples with the PCR. Appl. Environ. Microbiol.. 61: 116-121.
35. Neilan, B. A. (1995). Identification and phylogenetic analysis of toxigenic Cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. Appl. Environ. Microbiol.. 61: 2286-2291.
36. Li, X., Dorsch, M., Del Dot, T., Sly, L. I., Stackebrandt, E., Hayward, A.C. (1993). Phylogenetic studies of rRNA group II pseudomonads based on 16S rRNA gene sequences. J. Appl. Bacteriol. 74: 324-329.

Material may be protected by copyright law (Title 17, U.S. Code)