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Development of a PCR test for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*

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Abstract

A chromosomal DNA library of the bacterial pathogen of bean, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* NCPPB 559 was constructed in the plasmid pGEM-7Zf(+). Several clones were identified that hybridised to all *Curtobacterium flaccumfaciens* pathovars including: *C. f. betae*, *C. f. flaccumfaciens*, *C. f. oortii*, *C. f. poinsettiae* and, in addition, to some strains of *Clavibacter michiganensis* subsp. *insidiosus* and *Clavibacter michiganensis* subsp. One of these clones (pPMP-26), after subsequent digestion with restriction endonucleases *Eco*RI/*Sac*I, yielded a fragment of approximately 0.2 Kb (pPMP-26D) that hybridised specifically to *C. f. flaccumfaciens* and not to any of the other plant pathogenic members of the order *Actinomycetales* or any of the other prokaryotic bean pathogens tested. This fragment was subcloned and sequenced, analysis of the resultant 198 bp sequence showed that no significant homology existed with any other sequence currently deposited in public databases. Further analysis of these data facilitated the design of PCR primers which were subsequently tested against a wide range of plant pathogenic actinomycetes and other prokaryotic bean pathogens. Results show that these primers are highly specific for all strains of *C. f. flaccumfaciens* with no cross-reaction to strains from any other bacterial taxa tested.

Introduction

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (Hedges) Collins & Jones is the causal agent of bacterial wilt of beans. The pathogen is seed-borne and of phytosanitary significance (Calzolari et al. 1987; Smith et al. 1997). The bacterium has been reported to cause serious disease outbreaks in the United States (Thomas & Graham 1952; Dowson 1957; Coyne & Schuster 1979; Venette et al. 1995) and its presence has been recorded in diverse geographical areas within Europe, Australia, Asia, North and South America, and Africa (Bradbury 1986; Smith et al. 1997). The long latency period prior to the development of the disease, the relatively slow growth of the bacterium on complex media, its endophytic nature and occurrence in low numbers (Van Vuurde et al. 1983) have made disease diagnosis and pathogen detection difficult, especially in seed certification programmes or quarantine inspection of imports.

To date, a variety of different methods have been employed to circumscribe, identify and detect *Curtobacterium* spp., in particular *C. f. flaccumfaciens*. These include: numerical taxonomy (Jones 1975; Locci et al. 1989), identification kits (e.g. BIOLOG; Zhao et al. 1997), analysis of cell wall amino acids (Yamada & Komagata 1972), whole cell proteins (Carlson & Vidaver 1982), lipids (Collins et al. 1980; Collins & Jones 1980, 1983; Henningson & Gudmestad 1991) and polyamines (Altenburger et al. 1997). In addition, serology (Lazar 1968; Calzolari et al. 1987; Diatlof et al. 1993; Mizuno et al. 1995;

McDonald & Wong 2000), DNA homology (Starr et al. 1975), PCR fingerprinting methods (McDonald & Wong 2000) and 16S rDNA fingerprinting using temperature gradient gel electrophoresis (Felske et al. 1999) have also been used. In general, these methods can be used to confirm the identity of isolates at the genus- and species-level, but most lack the resolution to identify strains down to pathovar. As a consequence, the premise established by Vidaver & Starr (1981) still has credibility today in that, there is no easy or certain means to identify strains of phytopathogenic actinomycetes (coryneforms) below the species level, other than by host testing.

The present quarantine procedures used for the detection of *C. f. flaccumfaciens* in seed lots include visual inspection of the seeds and a slide agglutination test (Calzolari et al. 1987; Smith et al. 1997). Immunofluorescence staining has also been used to detect the bacterium in contaminated seed lots (Calzolari et al. 1987; Diatlof et al. 1993). None of these procedures have proved to be entirely effective on their own. Visual inspection cannot locate latent infections, whilst polyclonal antibodies are not effective against all strains of the pathogen (Calzolari et al. 1987; McDonald & Wong 2000). Although, the use of monoclonals has enhanced specificity and sensitivity, all procedures described to date are ineffective against all strains of the pathogen (Diatlof et al. 1993; Mizuno et al. 1995; McDonald & Wong 2000).

The development and preliminary testing of a PCR detection method described here presents the prospect of a fast, accurate and reliable diagnostic test for *C. f. flaccumfaciens*.

Materials and methods

Bacterial strains The bacterial strains used in this study are listed in Tables 1 and 2. Cultures were stored at −80 °C in Luria-Bertani medium (LB) containing 10% glycerol and grown in the same medium at 28 °C (Maniatis et al. 1982).

Construction of the chromosomal library *C.f. flaccumfaciens* NCPPB 559 was grown to mid-log phase on liquid LB medium, harvested by centrifugation and DNA extracted and purified (Maniatis et al. 1982). The DNA was digested with *Sau*3AI (New England Biolabs Inc.) to an average size of 5 Kb and the plasmid vector pGEM-7Zf(+) (Promega, Madison, WI) was used for the construction of the library. The ligation products were used to transform *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla), following the manufacturers instructions.

Construction of the chromosomal library Potential mutants were identified by screening transformed cells for acquisition of ampicillin resistance and a white colony colour on LB medium containing ampicillin (100 μg/ml, w/v), X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 40 μg/ml, w/v) and 40 μm IPTG (isopropylthio-β-D-galactoside).

Hybridisation Plasmid DNA was extracted from transformed colonies of *Escherichia coli* and digested with *Bam*HI and *Bst*XI. The plasmid DNA was separated by electrophoresis in a 1.5% agarose gel in Tris-borate buffer, denatured and transferred to nylon membranes (Hybond-N, Amersham) for Southern blotting (Southern 1975). Replica gels were made and the membranes were hybridised with total genomic DNA of *C. f. flaccumfaciens* or *Clavibacter michiganensis* (*Clim.*) subsp labelled with ^{32}P-dCTP by random primer labelling according to the supplier's instructions (Multiprimer DNA labelling, Amersham).

Probe DNA selected in Southern-blot analysis were excised from the pGEM vector using *Bam*HI/*Bst* XI double digests followed by gel purification using GENECLEAN (Bio/Can Scientific Inc. Mississauga, Ont.). These fragments were labelled with ^{32}P or digoxigenin-dUTP (Boehringer Manheim, UK) according to the supplier's instructions and used as probes in colony and dot-blots.

Restriction digests and subcloning Genomic fragments that hybridised to the *Curtobacterium* strains but not to members of the genera *Clavibacter* and *Rathayibacter* were treated with different restriction enzymes according to the supplier's instructions and the resulting fragments labelled with ^{32}P-dCTP or digoxigenin-dUTP prior to further screening against all strains. Fragments that were found to be specific for *C. f. flaccumfaciens* were subcloned in the same pGEM vector, as detailed above.

DNA probe sequencing Fragments were sequenced using the dideoxy-mediated chain termination method described by Sanger et al. (1977). DNA sequencing was carried out on an ABI 373 A DNA sequencer (Perkin Elmer/Applied Biosystems Division (Foster City, CA)). The sequence (GenBank Accession Number AF277098) was compared to sequence databases at the National Center for Biotechnology Information

Table 1. Strains of *Curtobacterium* used in the analysis

Species	Strain	Host	Origin
Curtobacterium flaccumfaciens pv. *betae*	[1]NCPPB 363, 364, 372, 373, 374^T, 375	*Betae vulgaris*	UK
Curtobacterium flaccumfaciens pv. *flaccumfaciens*	NCPPB 567	*Phaseolus vulgaris*	Germany
	NCPPB 14, 1412, 1435, 1441, 1442, 1446^T	"	Hungary
	NCPPB 1597, 1751	"	Romania
	NCPPB 178, 558, 559, 1843, 1844, 2343, 2344	"	U.S.A
	[2]IMI 350460	"	Hungary
	IMI 347350	"	Kenya
Curtobacterium flaccumfaciens pv. *oortii*	NCPPB 2944, 2945	*Tulipa gesneriana*	Japan
	NCPPB 2113^T, 2114	"	Netherlands
	NCPPB 2240, 2241, 2305	*Tulipa gesneriana*	UK
Curtobacterium flaccumfaciens pv. *poinsettiae*	NCPPB 177, 844, 845, 846, 847, 848, 849, 853, 854^T, 855	*E. pulcherrima*	USA
Curtobacterium albidium	ATCC 15831^T	–	Japan
Curtobacterium citreum	[3]ATCC 15828^T	–	Japan
Curtobacterium luteum	ATCC 15830^T	–	Japan
Curtobacterium plantarum	ATCC 49174^T	Glycine max	USA
Curtobacterium pusillum	ATCC 19096^T, 19097	–	Japan

[1]National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, UK.
[2]IMI, CABI Bioscience, Egham, Surrey, UK.
[3]American Type Culture Collection, Rockville, Md., USA.
^{T}Type culture

(NCBI) and the National Library of Medicine (NIH) (Bethesda, MD) using the BLAST algorithm (Altschul et al. 1990).

PCR detection Primers were designed using the Primer Designer Program (Version 2.0. Scientific and Educational Software, Durham, NC, USA). A number of primer sets were designed and tested and the forward and reverse primers (CF4 $^{5'}$CACAGCCACCTACATGC$^{3'}$ & CF5 $^{5'}$GATCGGGAGTCCGAG$^{3'}$) which produced optimal results, were subsequently used in detection assays. Five μl of bacterial DNA, extracted using methods described above were added to 45 μl of master mix (200 μM dNTP's, 0.2 μM primers, 1.25U Tth polymerase and the manufacturer's buffer). Amplification was achieved with the following programme: Initial denaturing at 96 °C for 2 min, then 94 °C for 30 sec denaturation, 55 °C for 30 sec annealing, 72 °C for 30 sec extension (for 34 cycles), then a final extension at 72 °C for 10 min. Amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light.

Table 2. Strains of other phytopathogenic actinomycetes and representative bacterial bean pathogens used in the analysis

Species	Strain	Host	Origin
Clavibacter michiganensis subsp. *insidiosus*	[1]IMI 347334	*Medicago sativa*	Australia
Clavibacter michiganensis subsp. *michiganensis*	[2]CNPH 02, 03, 05	*Capsicum annuum*	Brazil
	CNPH 08, 01	*Lycopersicon esculentum*	Brazil
	IMI 347346	"	Kenya
	IMI 347333	"	South Africa
	IMI 347344	"	U.S.A
	IMI 347332, 347345, 350455	"	Zambia
Clavibacter michiganensis subsp. *nebraskensis*	[3]NCPPB 2579, 2581^T, 2582	*Zea mays*	USA
Clavibacter michiganensis subsp. *tessellarius*	[4]SCHAAD 7818, 78141	*Triticum aestivum*	USA
Rathayibacter iranicus	NCPPB 2253^T	*Triticum aestivum*	Iran
Rathayibacter tritici	NCPPB 471	*Triticum aestivum*	India
	NCPPB 1857^T	"	Egypt
	NCPPB 1953	"	Iran
Erwinia carotovora subsp. *carotovora*	[5]UNB 01	*Solanum tuberosum*	Brazil
Pseudomonas cichorii	IMI 347490	*Phaseolus vulgaris*	Ireland
P. marginalis pv. *marginalis*	IMI 347524	*Phaseolus vulgaris*	Canada
P. syringae pv. *glycinea*	IMI 349379	*Phaseolus vulgaris*	U.K.
P. syringae pv.*phaseolicola*	IMI 349239	*Phaseolus vulgaris*	Canada
	IMI 349228	*Phaseolus vulgaris*	Uganda
P. syringae syringae	IMI 347447	*Phaseolus vulgaris*	France
Pseudomonas syringae pv. *tomato*	UNB 03	*Lycopersicon esculentum*	Brazil
X. campestris pv. *phaseoli*	IMI 349444	*Phaseolus vulgaris*	Uganda
X. campestris pv. *vignicola*	IMI 350023	*Phaseolus vulgaris*	India

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[2] Centro Nacional de Pesquisa de Hortalicas (CNPH/EMBRAPA), Brasilia, D.F., Brazil.
[3] National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, UK.
[4] Dr. Norman W. Schaad, Agricultural Research Center, USDA, USA.
[5] Universidade de Brasilia (Unb), Brasilia, D.F., Brazil.
^{T}Type culture.

Results

Development of the DNA probes from the C.f. flaccumfaciens chromosomal library Restriction digestion of a random sample of one hundred clones from the chromosomal library generated a diverse range of restriction fragments, with insert size varying from 5 to 1 Kb (data not shown). To identify potential specific fragments, Southern-blots of randomly selected library clones double digested with *Bam*HI/*Bst*XI were hybridised with total genomic DNA of *C.f. flaccumfaciens* NCPPB 559 and *Cl.m. michiganensis* CNPH 08. Twelve clones that hybridised only to *C.f. flaccumfaciens* were selected and used as probes to screen a wide range of bacteria. One of these clones, pPMP-26 produced strong hybridisation signals on colony-blots to the following species: *C.f. betae*, *C.f. flaccumfaciens*, *C.f. oortii*, *C.f. poinsettiae*, *Cl.m. insidiosus* and two strains of *Cl.m. michiganensis* (data not shown). In dot-blots containing approximately 200 ng of DNA from a range of bacteria, no hybridisation signals were observed with any of the *Clavibacter*, *Erwinia*, *Pseudomonas* or *Xanthomonas* strains. A weak hybridisation signal was observed with one *Rathayibacter* strain whilst most *Curtobacterium* strains produced strong hybridisation reactions (Figure 1).

Clone pPMP-26, with a DNA insert of 4.7 Kb, when double digested with *Eco*RI/*Sac*I produced four restriction fragments (26 A, 26 B, 26 C and 26 D) of 1.9, 1.6, 1.0 and 0.2 Kb respectively (Figure 2). The pPMP-26 D fragment hybridised strongly to *C.f. flaccumfaciens* in dot blots but did not show homology to any of the other twenty-two actinomycetes or bean pathogens (Figure 2). The other three fragments of the pPMP-26 (A, B, C) showed varying levels of homology to the strains of *Curtobacterium* (data not shown).

The pPMP-26 D fragment when labelled with digoxigenin-dUTP was extremely specific and showed homology only to the *C. f. flaccumfaciens* strains. No hybridisation signal was observed with representatives of *C.f. betae*, *C.f. oortii*, *C.f. poinsettiae*, *Cl.m. michiganensis, Rathyibacter iranicus*, *R. tritici*, *Pseudomonas cichorii*, *P. marginalis* pv. *marginalis*, *P. syringae* pv. *glycinea*, *P.s.* pv. *phaseolicola*, *P.s. syringae*, *Xanthomonas campestris* pv. *phaseoli* and *X.c. vignicola* tested on dot blots (Figure 3).

Probe sequencing and PCR detection The DNA fragment pPMP-26 D subcloned in the pGEM-7Zf(+) *Eco*RI/*Sac*I sites contained 198 bp (Figure 4). The nucleotide sequence search performed using the BLAST algorithm showed that the DNA probe had no significant homology to sequences deposited in public databases. A range of PCR primers were generated and tested against all strains included in this study (data not shown). In particular, amplification was achieved from all strains of *C.f. flaccumfaciens* when using the CF4 & CF5 primers, and not from strains of other taxa (Figure 5), including all other pathovars within the species *Curtobacterium flaccumfaciens*, other members of the genus *Curtobacterium*, other phytopathogenic actinomycetes and representative bacterial bean pathogens.

Discussion

The need for speed and accuracy are paramount in the detection of seedborne pathogens. Historically, bacterial seedborne pathogens have been detected by 'growing-on' seeds under controlled conditions and noting the emergence of disease symptoms (Maude 1996). Direct isolation of pathogens has also been widely used, and has the advantage that pure cultures of the suspected pathogen can be identified and their pathogenicity subsequently confirmed. These methods, whilst still in use, have a number of drawbacks namely that they are time consuming and in the case of the former, may require a great deal of space and other resources. The direct isolation approach is open to interference from non-target organisms, particularly if pathogen numbers are low (Schaad et al. 1997; Van Vuurde 1997). Serological methods have been in use for a number of years and are generally simple to perform, rapid and accurate when used to detect a number of bacterial pathogens (Schaad 1979; Ball & Reeves 1992). However, some serological methods, in particular ELISA lack sensitivity (Van Vuurde 1997) and in certain cases may give ambiguous results (Slack et al. 1996; Schaad et al. 1997).

A variety of serological approaches have been described for the identification and detection of *C.f. flaccumfaciens* (Calzolari et al. 1987; Diatlof et al. 1993; Mizuno et al. 1995; Smith et al. 1997; McDonald & Wong 2000) and these have been applied either directly to environmental samples or subsequently to isolation of colonies from plant materials. All methods described to date suffer a major drawback in that they are unable to detect all strains of the pathogen and are therefore limited in their use.

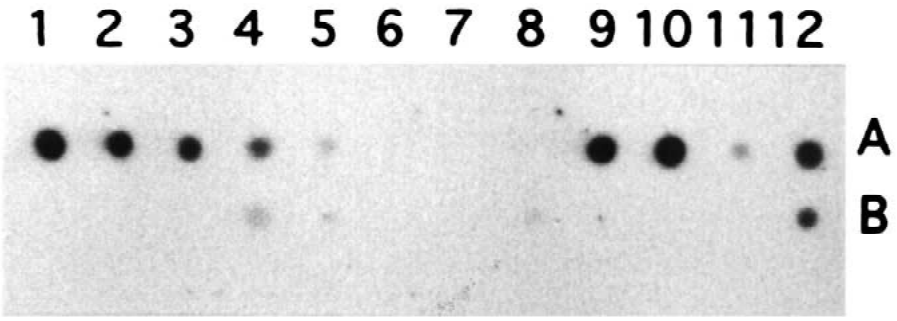

Figure 1. Dot blot hybridization of total DNA of some phytopathogenic actinomycetes and bean seedborne pathogens probed with *C.f. flaccumfaciens* library clone pPMP-26. Lane A, *C.f. flaccumfaciens* NCPPB 559 (1), 558 (2), *C.f. poinsettiae* NCPPB 844 (3), 854 (4), 855 (5); *R. tritici* NCPPB 471 (6), 1857 (7), 1953 (8), *C. f. oortii* NCPPB 2113 (9), 2945 (10), 2114 (11), *C.f. betae* NCPPB 375 (12); Lane B, *Cl.m. nebraskensis* NCPPB 2579 (1), 2581 (2), 2582 (3); *R. iranicus* NCPPB 2253 (4), *Cl. m. michiganensis* CNPH 08 (5), 01 (6), IMI 347344 (7), 347345 (8), *E. carotovora* subsp.*carotovora* UNB 01 (9), *X.c. phaseoli* IMI 349444 (10), *P.s. syringae* IMI 347447 (11), *C.f. flaccumfaciens* NCPPB 559 (12).

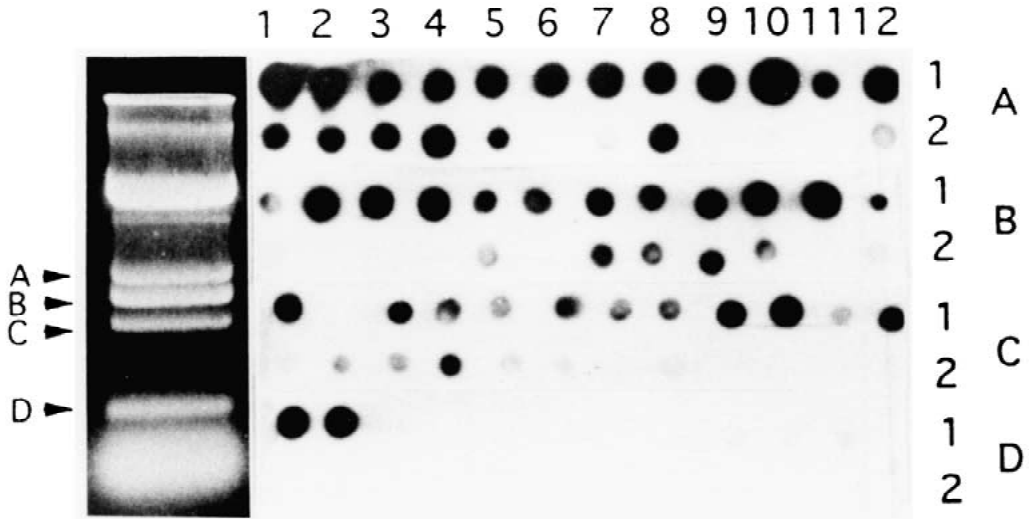

Figure 2. Gel electrophoretic analysis of *Eco*R1/*Sac*1 restriction digestion of clone pPMP-26 alongside dot blot analysis of total DNA of some phytopathogenic actinomycetes and other bacterial phytopathogens probed with the four different fragments of clone pPMP26 (A, B, C, D). Lane 1, *C.f. flaccumfaciens* NCPPB 559 (1), 558 (2), *C.f. poinsettiae* NCPPB 844 (3), 854 (4), 855 (5), *R. tritici* NCPPB 471 (6), 1857 (7), 1953 (8), *C.f. oortii* NCPPB 2114 (9), 2113 (10), 2945 (11), *C.f. betae* NCPPB 375; Lane 2, *Cl.m. nebraskensis* NCPPB 2579 (1), 2581 (2), 2582 (3), *R. iranicus* NCPBB 2253 (4), *Cl.m. michiganensis* IMI 347332 (5), CNPH 01 (6), CNPH 02 (7), IMI 347344 (8), *E.c. carotovora* UNB 01 (9), *X.c. phaseoli* IMI 349444 (10), *P.s. tomato* UNB 03 (11), *Cl.m. insidiosus* IMI 347334 (12).

Increasingly, PCR detection methods are being used in the identification and detection of bacterial phytopathogens. Their applicability is undoubtedly at its greatest in seed certification and quarantine inspection, where sensitivity is a prerequisite and differentiation is frequently required at infraspecific levels. Various strategies have already been used to develop PCR primers for the identification and detection of phytopathogenic actinomycetes. Strategies include targeting the intergenic transcribed spacer region (Li & De Boer 1995; Pan et al. 1998), 16 S rDNA (Lee et al. 1997), insertion element IS*1121* (Lee et al. 1997), plasmid fragments (Schneider et al. 1993; Hu et al. 1995) and plasmid borne inverted repeat sequences (Slack et al. 1996). However, some of these targets and strategies lack the resolution to differentiate between closely related strains, this is particularly true for detection systems based on 16S rDNA genes, which require other tests (Li et al. 1997) or further digestion of the amplification product (Lee et al. 1997) to confirm strain identity down to the pathovar level.

The majority of PCR methods described to date for the detection of phytopathogenic actinomycetes have concentrated on members of the genus *Clavi-*

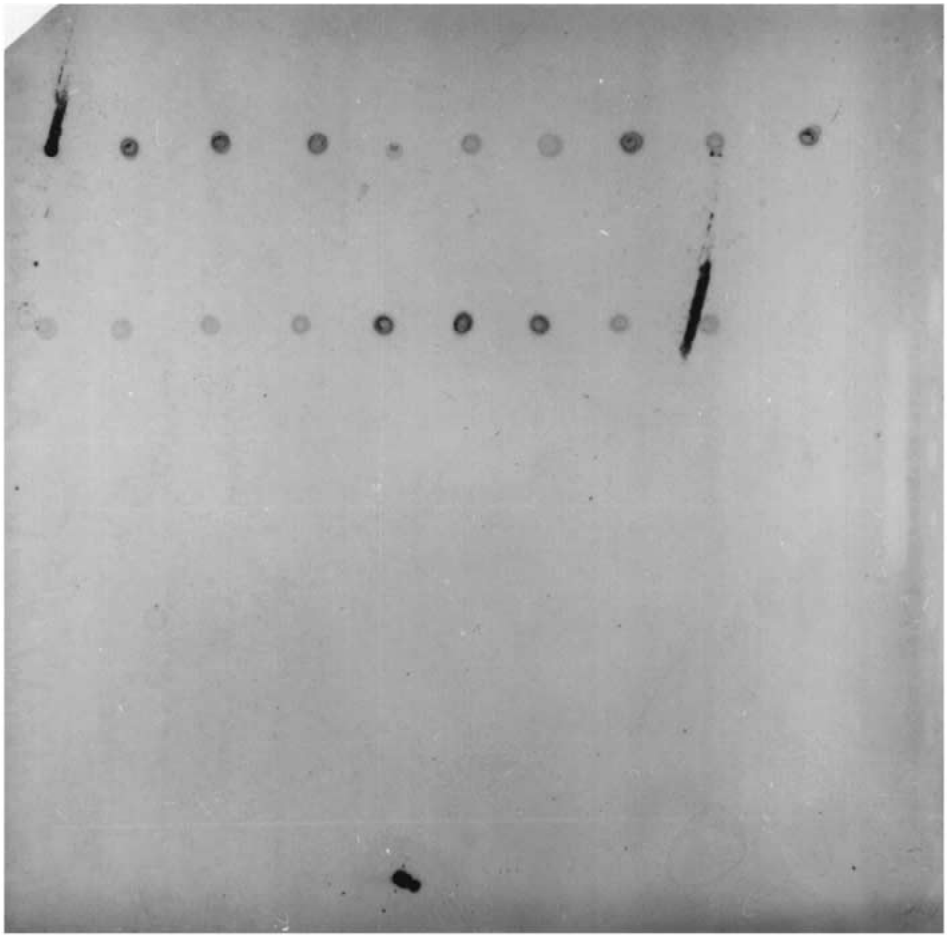

Figure 3. Dot blot of fifty-six strains of phytopathogenic actinomycetes and other bean pathogens probed with pPMP-26 D fragment labelled with digoxigenin-dUTP. Row 1, positive control (pBR328 DNA, labelled with digoxigenin), *C.f. flaccumfaciens* NCPPB 559, 558, IMI 350460, 347350, NCPPB 1446, 1751, 1412, 14, 178; Row 2, *C.f. flaccumfaciens* NCPPB 567, 1435, 1441, 1442, 1597, 1843, 1844, 2343, 2344, *C. albidium* ATCC 15831; Row 3, *C. citreum* ATCC 15828, *C. luteum* ATCC 15830, *C. pusillum* ATCC 19096, 19097, *C.f. poinsettiae* NCPPB 854, 177, 844, 845, 846, 847; Row 4,*C.f. poinsettiae* NCPPB 848, 849, 853, 855, *C. f. betae* NCPPB 374, 363, 364, 372, 373, 375; Row 5, *C.f. poinsettiae* IMI 347347,*C.f. oortii* NCPPB 2945, 2114, 2113, 2240, 2241, 2241, 2305, 2944, *X. c. phaseoli* IMI 349444; Row 6, *P.m. marginalis* IMI 347524, *P.s. syringae* IMI 347447, *P.s. phaseolicola* IMI 349239, *P. cichorii* IMI 347490, *P.s. phaseolicola* IMI 349228, *X.c. vignicola* IMI 350023, *P.s. glycinea* IMI 349379.

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1    5' GCACAGCCAC CTACATGCCG ATCAGCGCCG ATCAGGCCGC CCGGCAGCTT

51   CCGAACCTGC AGAAGGTCAG CGCCAAGACC GCCGGGTGGC TGCTCGAGGA

101  CTCACCTCG AGCGCCACTG CAACCGGCGC CGAGCTCGGA CTCCCGATCG

151  TCACCGCCGC ACGCCCCACG ACAGAGGCGC AGGTTATCGA GATCCGGA 3' 198
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Figure 4. Nucleotide sequence of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* NCPPB 559 genomic library clone pPMP-26 D. GenBank accession number AF277098. Primer annealing sites are underlined.

bacter. Extensive analysis of strains within the genus *Curtobacterium* and *Curtobacterium flaccumfaciens*, in particular, within our laboratory (unpublished data) and from the literature (McDonald & Wong 2000) highlights a very high degree of homogeneity within the species. In the case of the latter study, fingerprinting using primers targeted against repetitive genomic elements failed to fully resolve the pathovars. These findings would suggest that a conventional approach to bacterial differentiation and detection based on differ-

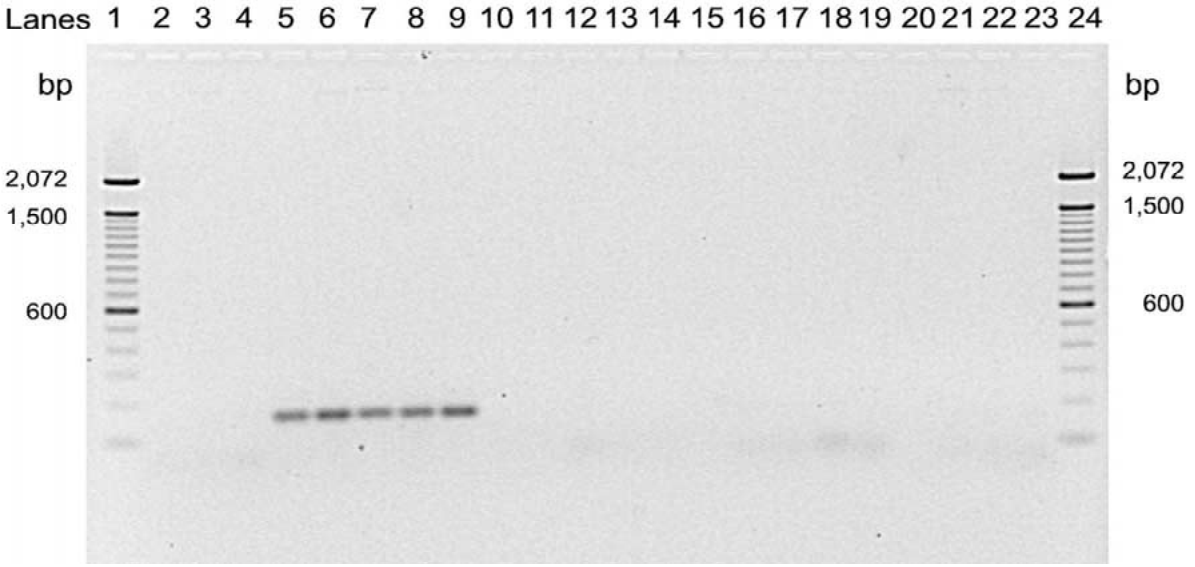

Figure 5. PCR amplification of some phytopathogenic actinomycetes and non-pathogenic members of the genus *Curtobacterium* using PCR primers CF4 & CF5. Lanes 1 & 24, 100 bp ladder (GibcoBRL); 2-4, *Cl.m. michiganensis* IMI 347332, 347345, 350455; 5-9, *C.f. flaccumfaciens* NCPPB 559, 1412, 567, 1441, 2343; 10-12, *C.f. poinsettiae* NCPPB 844, 177, 849; 13-15, *C.f. oorti* NCPPB2305, 2113, 2945; 16 & 17, *C.f. betae* NCPPB 363, 364; 18, *C. luteum* ATCC 15830; 19, *C. pusilum* ATCC 19096; 20, *C. plantarum* ATCC 49174; 21, *C. citreum* ATCC 15828; 22. *C. albidum* ATCC 15831; 23. Water control.

ences in the 16S rDNA or ITS sequence may lack the resolution to provide a robust test capable of detecting *C.f. flaccumfaciens* from other pathovars within the same species and, possibly other members of the genus. The approach we describe here makes no *a priori* judgements as to what genetic element would serve as a good target for pathovar delineation, it is based purely on observed differences with a closely related organism. The method is reliant on the detection of genomic fragments in *Curtobacterium* that were not detected in the closely related genus *Clavibacter*. A DNA sequence from *C.f. flaccumfaciens* that was absent from *Cl.m. michiganensis* was identified by hybridising Southern-blots of the genomic library with the DNA from both pathogens and selecting fragments of *C.f. flaccumfaciens* which did not hybridise with *Cl.m. michiganensis*. Subsequent restriction digestion of these fragments showed that as size decreased, the specificity towards *C.f. flaccumfaciens* was enhanced. The 0.2Kb fragment from clone pPMP-26 was extremely specific to all 18 strains of *C.f. flaccumfaciens*. It did not hybridise in dot or colony blots with any of the 60 other actinomycetes or other bean seed borne pathogens tested. The findings presented here have similarities to those of Thompson and co-workers (1989), who found that subsequent digestion of some clones drawn from a chromosomal library yielded probes which were highly specific for strains of *Cl.m. michiganensis*. Using a similar approach it was even possible to distinguish between virulent and avirulent forms of the pathogen.

In the study described here, sequencing of the fragment facilitated the logical design of PCR primers which were extensively studied against a range of phytopathogenic actinomycetes and other bean associated bacteria. Amplification was achieved from all 18 strains of *C.f. flaccumfaciens* and from no other bacteria contained within this study. Further testing of the primers showed no drop off in sensitivity when using boiled cells rather than extracted DNA (data not shown). Similar findings were also obtained using a subtractive hybridisation approach to develop a detection assay for *Cl.m. michiganensis* (Mills et al. 1997). Sequencing of the identified fragments yielded PCR primers which showed a high degree of specificity and sensitivity was assessed down to 100 CFU per millilitre.

Clearly, the primers described here require further testing before they can be used in a quarantine situation and, in particular, they need to be fully tested out with contaminated seed material. This is required to ensure that the method is not disrupted by the presence of inhibitory compounds originating from plant materials. This phenomenon is well known and has

resulted in the production of false negative reactions in other methods described to detect bacterial phytopthogens in seed and plant samples (Rasmussen & Wulff 1990). In such cases a two-pronged approach has usually been adopted involving the use of PCR and colony hybridisation, simultaneously, to rapidly identify and eliminate false negative results (Rasmussen & Reeves 1992). Such an approach could be adopted with the system described here. More recently however, a highly sensitive procedure for detecting seedborne bacteria has been described, BIO-PCR, which introduces a short, colony enrichment step prior to the PCR reaction (Schaad et al. 1997). This approach, like that described by Rasmussen & Reeves (1992), provides improvements over PCR detection used alone as it eliminates the risk of false negative and false positive reactions, the latter from the presence of dead or inactive cells. However, BIO-PCR provides the additional advantage of enhanced sensitivity. An increase in sensitivity of 100 fold has been described when compared against conventional PCR.

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