European Journal of Plant Pathology **109:** 817–825, 2003.

Characterisation of *Curtobacterium flaccumfaciens* pathovars by AFLP, rep-PCR and pulsed-field gel electrophoresis

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Accepted 18 March 2003

Key words: AFLP, genomic fingerprinting, *Curtobacterium flaccumfaciens*, PFGE, rep-PCR

Abstract

The bacterial species *Curtobacterium flaccumfaciens* encompasses a group of closely related phytopathogens subdivided into pathovars that exhibit differences in host range. To date, reliable differentiation of pathovars and identification of unknown isolates to the pathovar-level can only be achieved on the basis of host testing. In this study, representative strains from *C. flaccumfaciens* pathovars and related species were examined using a range of genomic fingerprinting techniques to ascertain their application as additional or potential alternatives to host testing. Amplified fragment length polymorphism (AFLP) and repetitive sequence polymerase chain reaction (rep-PCR) analyses enabled the categorisation of some, but not all strains, in their respective pathovars. In contrast, all strains were correctly assigned to pathovars using *Hin*dIII and *Xba*I macro-restriction digests in conjunction with pulsed-field gel electrophoresis (PFGE). Fingerprints generated by PFGE not only supported the current pathovar rankings within *C. flaccumfaciens*, they also identified subgroups within pathovars *flaccumfaciens*, *oortii* and *poinsettiae*.

Introduction

The classification of plant pathogenic Gram positive bacteria, which historically have been referred to as 'coryneforms', has undergone major revision in recent years. Currently the majority of this artificial group, which excludes other Gram positive phytopathogens such as the filamentous streptomycetes and the wall-less phytoplasmas, are recovered in the genera *Arthrobacter*, *Clavibacter*, *Curtobacterium*, *Leifsonia*, *Nocardia*, *Rathayibacter* and *Rhodococcus*. Despite major advances in our understanding of the inter-relatedness of species within this group and the description of their diversity, differentiation and identification of some closely related taxa remains problematic.

Amongst *Curtobacterium* species, *C. flaccumfaciens* comprises all the plant pathogens within this genus. This species was subdivided into four pathovars; *C.f.* pv. *betae*, *C.f.* pv. *flaccumfaciens*, *C.f.* pv. *oortii* and *C.f.* pv. *poinsettiae* (Bradbury, 1986), with a further pathovar, *C.f.* pv. *basellae,* causing leafspot on malabar spinach described by Chen et al. (2000). These pathovars show a high degree of host-specificity and inter-relatedness. Host-specificity testing remains the most effective means of subspecific differentiation because other methods, including biochemical tests (Harris-Baldwin and Gudmestad, 1996; Zhao et al., 1997), fatty acid profiles (Henningson and Gudmestad, 1991), protein profiles (Carlson and Vidaver, 1982), temperature-gradient gel electrophoresis analysis of 16S rRNA genes (Felske et al., 1999), numerical phenetic analysis (Dye and Kemp, 1977), cell wall analysis (Keddie and Cure, 1977; Davis, 1986) and serology (McDonald and Wong, 2000), revealed few identifiable differences among pathovars.

Differences in host range are pivotal characteristics in the characterisation of closely related phytopathogenic bacteria (Dye et al., 1980; Young et al., 1991). Despite the benefits associated with constructing classifications built on the ability of groups of strains to cause plant disease, there is a requirement to develop more rapid, additional or alternative methods to add weight or validate classifications and to facilitate rapid identification. Genomic fingerprinting methods based upon the polymerase chain reaction (PCR), such as amplified fragment length polymorphism (AFLP; Vos et al., 1995), repetitive DNA sequence-PCR (rep-PCR; de Brujin, 1992) and rDNA intergenic spacer regions (Jensen et al., 1993) analyses, have generated insights into the relatedness of a large number of closely related clinically and environmentally significant bacteria. These methods have been used to study a wide range of bacterial phytopathogens revealing levels of genetic diversity previously unrealised (Thwaites et al., 1999; Rademaker et al., 2000). In a comprehensive study of *C. michiganensis* subspecies *sepedonicus*, Smith et al. (2001) found that this subspecies was easily distinguishable from other subspecies using BOX–PCR and that the method offered a credible alternative to an eggplant bioassay, the existing method for identifying fresh isolates of the pathogen to the subspecies. In a similar study of *C.f.* pv. *flaccumfaciens* and its relatives, rep-PCR was found to be effective at separating the species from other Gram positive pathogens but could not fully resolve strains of this pathovar from representative of other pathovars of *C. flaccumfaciens* (McDonald and Wong, 2000). In addition, *C.f.* pv. *flaccumfaciens* strains were recovered in two clusters, a finding which was congruent with results obtained using a number of specific monoclonal antibodies.

In addition to PCR-based methods, the use of rare-cutting restriction endonucleases in conjunction with fragment resolution using pulsed-field gel electrophoresis (PFGE) has proven to be of great value in a number of epidemiological studies (Smith et al., 1995; Hanninen et al., 2001; Klaassen et al., 2002). The method has been shown to be particularly powerful and of relevance to studies of plant disease epidemiology and the differentiation of close relatives.

In this study genomic fingerprints generated by AFLP, rep-PCR and PFGE were evaluated as a potential means of pathovar characterisation in *C. flaccumfaciens*.

Table 1. List of strains included in this study

Species	Strain number*	Host (if known)	Origin
Curtobacterium flaccumfaciens pv. *betae*	347347*	*Betae vulgaris*	Canada
	347349, 347351, 350459^T, 367323, 367324, 367325, 367326, 367327	*Betae vulgaris*	UK
C.f. pv. *flaccumfaciens*	350460, 358455, 367330, 367331, 367332	*Phaseolus vulgaris*	Hungary
	347350	*Phaseolus vulgaris*	Kenya
	367334	*Phaseolus vulgaris*	Romania
	367329	*Phaseolus vulgaris*	Germany
	347348, 358450, 358451, 367328, 367333, 367335, 367336, 367337, 367338	*Phaseolus vulgaris*	USA
C.f. pv. *oortii*	350461,	*Tulipa gesneriana*	Netherlands
	367339, 367340, 367341	*Tulipa gesneriana*	UK
	367342, 358464	*Tulipa gesneriana*	Japan
C.f. pv. *poinsettiae*	350462, 358452^T, 358453, 358454, 367343, 367344, 367345, 367346, 367347, 367348, 367349, 367350	*Euphorbia pulcherrima*	USA
C. citreum	359423^T	—	Japan
C. albidium	359422^T	—	Japan
C. luteum	359424^T	—	Japan
C. pusillum	359425^T	—	Japan
C. plantarum	359426^T	*Glycine max*	USA

*Unless otherwise stated all cultures supplied by The Genetic Resource Collection, CABI BIoscience, Egham, Surrey, UK.
T: Type culture.

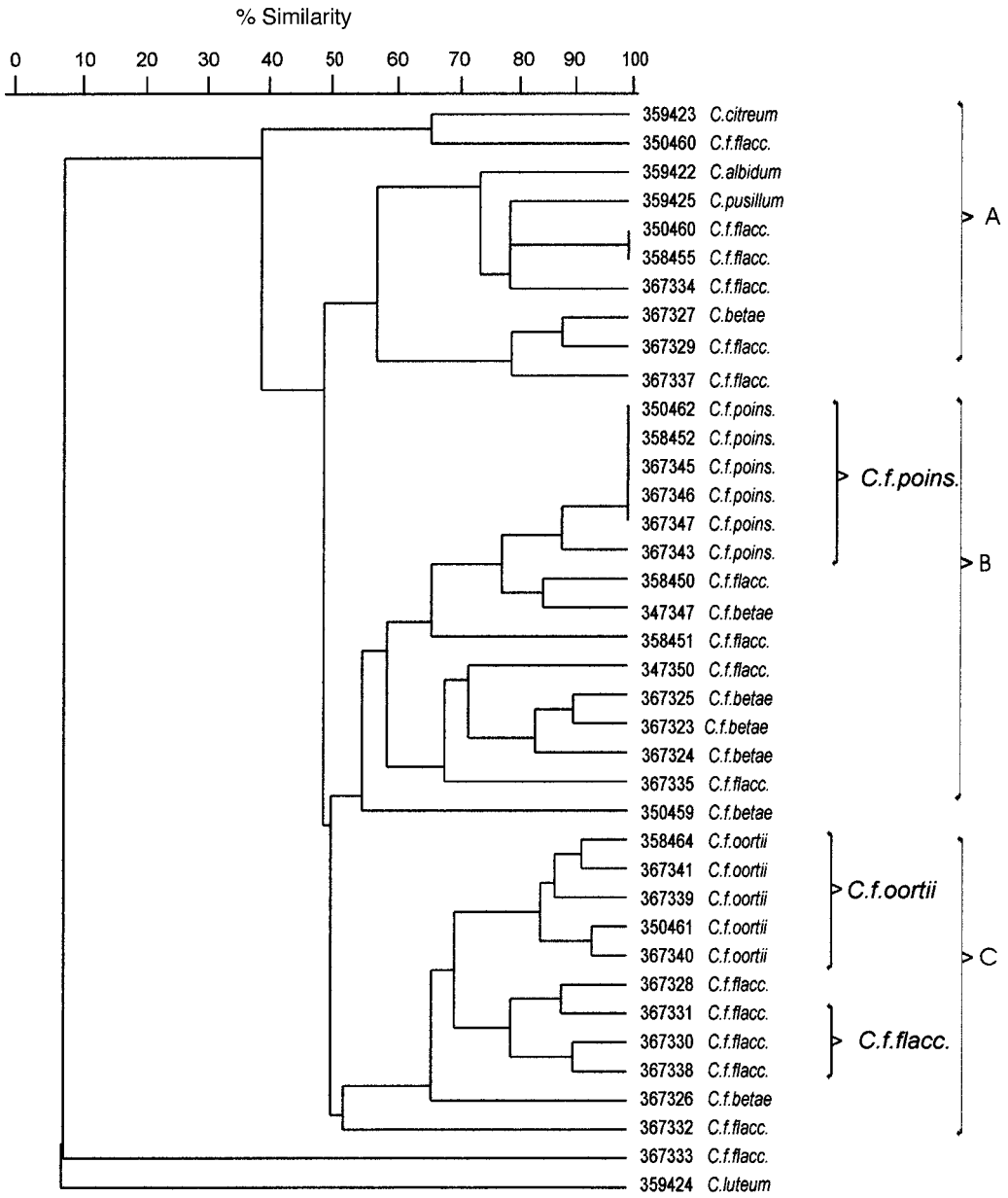

Figure 1. Dendrogram of BOX–PCR fingerprint data. Similarities were defined using Dice's coefficient and clustering using UPGMA. Details of all strains are as described in Table 1.

Materials and methods

Bacterial cultures

Details of *Curtobacterium* strains used in this study are summarised in Table 1. The identity of all cultures was confirmed to the species-level using whole-cell fatty acid methyl-ester analysis using the Microbial Identification System (MIS; MIDI Inc., Newark, DE, USA). Cultivation, extraction and analysis conditions have been described previously (Henningson and Gudmestad, 1991). The bacterial cultures were

maintained freeze-dried and grown overnight for DNA extraction on liquid CPG (Casamino acids (DIFCO) 0.1% (w/v), Bacto peptone (DIFCO) 1% (w/v) and glucose 0.5% (w/v)) or on TSBA (Tryptcase soy broth (BBL) 3% (w/v), agar 1.5% (w/v)).

DNA extraction and PCR amplification

Total genomic DNA was prepared using the procedure described by Graves and Swaminathan (1993). rep-PCR was performed using the primer sequence corresponding to BOX A, a subunit of the BOX element (5′-CTACGGCAAGGCGACGCTGACG-3′; Martin et al., 1992) according to Louws et al. (1995). The AFLP method applied was described previously by Mueller et al. (1996), with bacterial DNA being simultaneously digested with *Pst*1 and ligated to corresponding double stranded adapters with two base selectivity *Pst*1 + GA/*Pst*1 + CA. PCR amplified products were separated by gel electrophoresis on 1.5% agarose gels in TAE buffer and visualised by examining ethidium bromide stained gels under UV light. Extractions and amplifications were duplicated and irreproducible peaks (bands) were omitted from the subsequent analysis of the genomic fingerprints.

Pulsed-field gel electrophoresis

The method used for the preparation of high molecular weight DNA was as described by Correia et al. (1994) with a lysozyme treatment conducted for 6 h at 37 °C followed by a 72 h proteinase K treatment at 56 °C. Genomic digestion with *Xba*I and *Hin*dIII was carried out in the presence of 10 U of restriction enzymes. Separation of DNA macrofragments was performed in 1.5% agarose gels in a LKB 2015 Pulsaphor Plus apparatus (Pharmacia) equipped with a hexagonal electrode array at 200 V with an interval time (linear ramp) from 3 to 30 s for 22 h. Bands were visualised by examining ethidium bromide stained gels under UV light. Extractions, digestions and analyses were duplicated and any irreproducible bands were omitted from the subsequent analysis of the genomic fingerprints.

Analysis of genomic fingerprints

DNA fingerprints were scored visually or directly using GelCompar (Applied Maths, Sint-Martens-Latem, Belgium). AFLP and rep-PCR were analysed directly from gel images, whilst PFGE data was scored visually and the presence/absence of bands collated into a binary data matrix. Cluster analysis for all fingerprint types was performed on similarity matrices produced using Dice's coefficient ($(2a/(2a + b + c)$); Dice, 1945), clustering achieved with the unweighted pair-group method with arithmetic averaging (UPGMA; Sneath and Sokal, 1973). Cluster analysis of PFGE data is supported by cophenetic correlation values, calculated using the method of Sokal and Rohlf (1962).

Results

The dendrogram produced from the analysis of the repetitive BOX element by PCR is shown in Figure 1. Strains were recovered in three major clusters linked at a similarity level of 48–57%. The first cluster (A) consisted of *C. citreum*, *C. albidium*, *C. pusillum*, *C.f.* pv. *flaccumfaciens* and *C.f.* pv. *betae*. The second (B) consisted of *C.f.* pv. *flaccumfaciens*, *C.f.* pv. *poinsettiae* and *C.f.* pv. *betae* with all strains of *C.f.* pv. *poinsettiae* clustered in one subgroup at a similarity value of 88%. The third group (C) consisted of all *C.f.* pv. *oortii* strains recovered at a similarity level of 85%, linked to a number of strains of *C.f.* pv. *flaccumfaciens* and *C.f.* pv. *betae*. The use of BOX primers allowed the differentiation of *C.f.* pv. *poinsettiae* and *C.f.* pv. *oortii* from the other *C. flaccumfaciens* pathovars. However, both *C.f.* pv. *flaccumfaciens* and *C.f.* pv. *betae* strains were widely dispersed across the dendrogram and there was

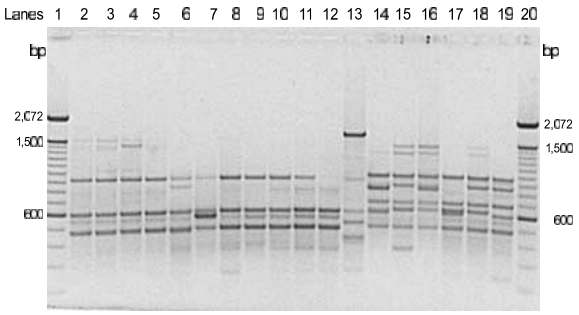

Figure 2. Example of a simplified AFLP analysis gel obtained using *Pst*1-specific adaptors and primers. Lanes: (1) 100-bp ladder (GibcoBRL); (2–13) *C.f.* pv. *poinsettiae* IMI 358452, 358454, 367343, 367345, 367350, 358453, 367344, 350462, 367348, 367347, 367346, 367349; (14–19) *C.f.* pv. *oortii* IMI 367341, 350461, 358464, 367342, 367339, 367340; (20) 100-bp ladder.

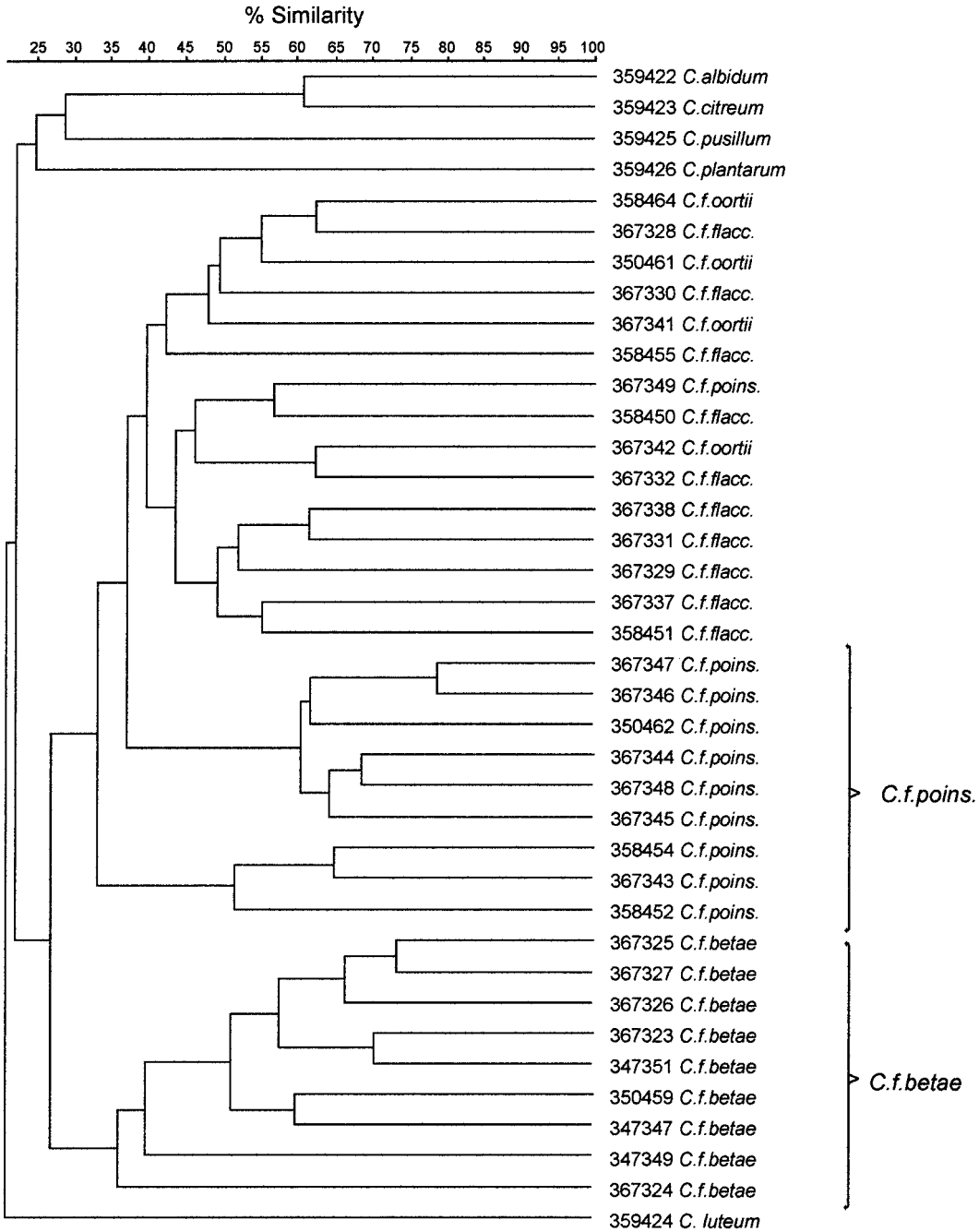

Figure 3. Dendrogram of AFLP fingerprint data. Similarities were defined using Dice's coefficient and clustering using UPGMA. Details of all strains are as described in Table 1.

no clear separation between *C. flaccumfaciens* and the majority of other *Curtobacterium* species studied.

An example of the fingerprints obtained using the simplified *Pst*1-AFLP method, employing the *Pst*1 + GA/*Pst*1 + CA selective primer extensions is shown in Figure 2. In general, 4–8 bands ranging approximately from 1800 to 300 bp were generated from all the strains studied. A composite data-set,

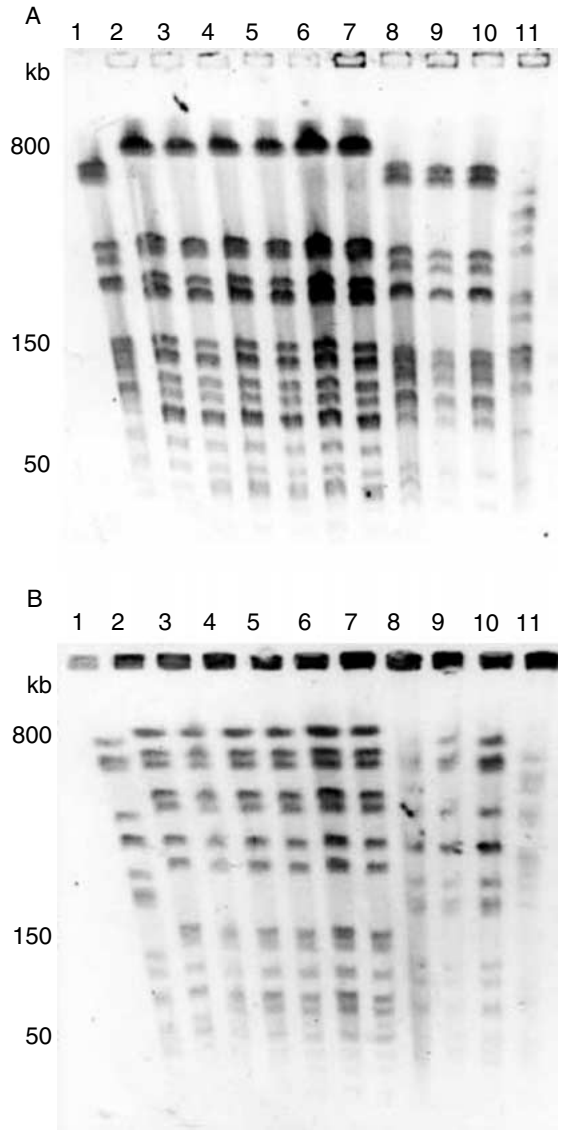

Figure 4. Pulsed-field gel electrophoresis of total genomic DNA of *Curtobacterium* spp. digested with *Hin*dIII (A) and *Xba*I (B). Lanes: (1–10) *C.f.* pv. *poinsettiae* IMI 367343, 367344, 367345, 367346, 367347, 367348, 367349, 367352, 350462, 358454; (11) *C. citreum* 359423.

constructed for all strains using two *Pst*1-specific adaptor/primer combinations was analysed and results shown in the form of a dendrogram (Figure 3). In contrast to the results obtained with the BOX primer, the five *Curtobacterium* species tested were well separated from each other. In addition, this method allowed the differentiation of *C.f.* pv. *poinsettiae* and *C.f.* pv. *betae* from representatives of the other pathovars.

Curtobacterium strains examined by macro-restriction analysis and PFGE generated DNA profiles consisting of 14–18 fragments, ranging from 5 to 800 Kb (Figure 4). Within *C. flaccumfaciens*, characteristic profiles for each pathovar were observed. Figure 4A,B shows an example of the electrophoretic patterns obtained, with 10 strains of *C.f.* pv. *poinsettiae* exhibiting two basic profile types. *C.f.* pv. *flaccumfaciens* strains digested with *Hin*dIII and *Xba*I showed a high degree of heterogeneity, with 5 different profiles types identified, *C.f.* pv. *oortii* strains exhibited two profile types and, in contrast, *C.f.* pv. *betae* displayed remarkably little heterogeneity in its macro-restriction profile (data not shown). The dendrogram generated from these collective data separated *C. flaccumfaciens* strains into four cluster groups with similarity values ranging from 28% to 43% (Figure 5). Membership of these four clusters was entirely consistent with the existing subspecific classification for this species and all strains were recovered in their respective pathovars, though *C. citreum* was found to cluster with strains of *C.f.* pv. *poinsettiae*. High values for cophenetic correlation (100–62%) at each of the branch points in the dendrogram indicate that the clustering is a good representation of the variation found within the similarity matrix.

Discussion

In general the differentiation of plant pathogenic coryneform bacteria remains problematic, this is particularly true of *C. flaccumfaciens* where the biochemical, serological and molecular approaches examined to date have proved inadequate at differentiating between pathovars (Henningson and Gudmestad, 1991; McDonald and Wong, 2000). In the study of McDonald and Wong (2000) genomic fingerprinting using rep-PCR, which includes BOX-, ERIC- and rep-PCR, was effective at separating *C. flaccumfaciens* from other species of the genus as well as from representatives of the genera *Clavibacter* and *Rathayibacter*. However, a number of *C.f.* pv. *flaccumfaciens* strains were recovered in one cluster alongside a *C.f.* pv. *poinsettiae* strain and others were clustered with strains of *C.f.* pv. *betae* and *C.f.* pv. *oortii*. The findings of this earlier research are mirrored here as only partial recovery of pathovars was achieved using BOX-PCR (Figure 1).

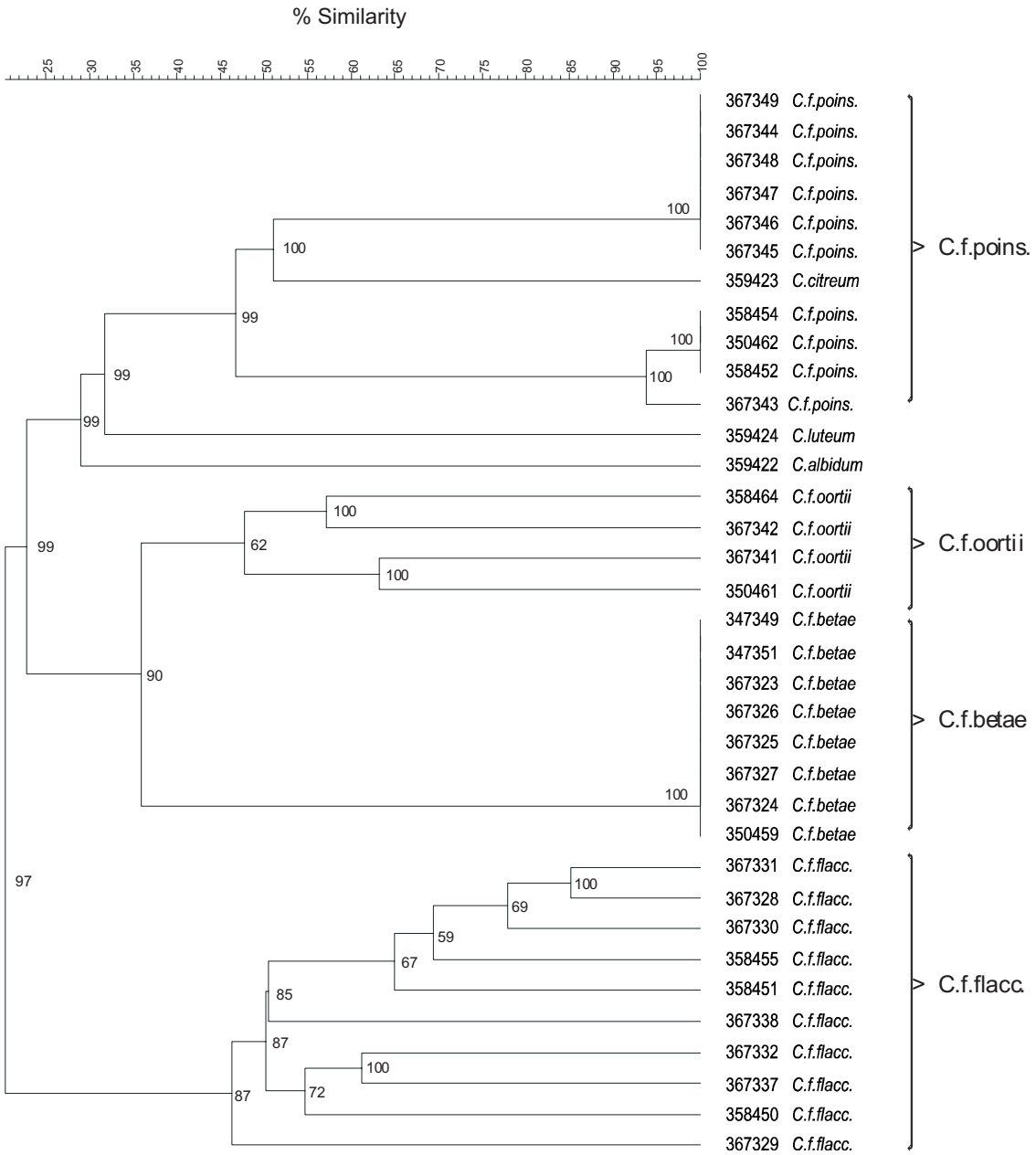

Figure 5. Dendrogram of PFGE fingerprint data with cophenetic correlation values shown at branch points. Similarities were defined using Dice's coefficient and clustering using UPGMA. Details of all strains are as described in Table 1.

In contrast to the work of McDonald and Wong (2000), *C. flaccumfaciens* could not be completely separated from other *Curtobacterium* species and only *C.f.* pv. *poinsettiae* and *C.f.* pv. *oortii* were recovered intact. The resolution obtained with this technique when compared with analogous methodology such as the restriction digestion of the ITS region (data not shown), is certainly superior though clearly not entirely effective at separating the pathovars of this species. These results and those from the earlier study

of McDonald and Wong (2000) contrast with studies on other closely related coryneforms, namely the subspecies of *Clavibacter michiganensis* (Louws et al., 1998; Smith et al., 2001).

Cluster analysis of the AFLP fingerprints (Figure 3) showed a higher level of resolution than BOX-PCR, separating *C. flaccumfaciens* from all other species of the genus tested and recovering *C.f.* pv. *poinsettiae* and *C.f.* pv. *betae* and a core of *C.f.* pv. *flaccumfaciens* as well separated groups. It is likely that the higher resolution obtained with this technique derives from restriction sites which are generally well dispersed across coding and non-coding regions of the genome enabling differentiation down to the strain-level in some bacteria (Blears et al., 1998; Loreti et al., 2001). AFLP analysis has the additional benefit of being relatively of free of artefacts (Hanninen et al., 2001), can be used effectively on bacterial species which have proven to be untypeable using other methods (Klaassen et al., 2002) and is amenable to computer-assisted analysis (de Boer et al., 2000). Clearly the simplified AFLP method used here generates high quality data with adequate resolution and time-efficiency, and may be an effective alternative for the characterisation and identification of some *C. flaccumfaciens* pathovars. However, it should be stressed that the method developed by Mueller et al. (1996) uses one restriction endonuclease rather than the conventional two and generates much simpler profiles than would normally be the case when using 'conventional' AFLP (Vos et al., 1995). This simplified method has the advantage that fragments can be separated and visualised by agarose-gel electrophoresis, though resolution maybe lost through this simplification of the method and the possibility exists that conventional AFLP may allow the full resolution of all pathovars of this species.

The macro-restriction digestion of genomic DNA and resolution of the resultant fragments by PFGE resulted in the clear differentiation of all *Curtobacterium* species, and the recovery of all strains in their respective pathovars. Varying numbers of subgroups were detected within each pathovar. Like AFLP's, the resolving power of PFGE, is reliant on the presence or absence of specific restriction sites across the genome, which can vary between individuals due to genomic rearrangement such as insertions, deletions, translocations or inversions. Furthermore, the distribution of these restriction sites in the bacterial genome is unique, stable, clonally inherited, and with the application of PFGE facilitates their direct observation (Hanninen et al., 2001). DNA fingerprints generated by PFGE have been used as diagnostic tools in the identification of microorganisms and in environmental studies (Hanninen et al., 2001), and as demonstrated here may provide an excellent alternative to host testing employed in *C. flaccumfaciens* pathovar identification.

The data produced in this study strongly support the idea that *C. flaccumfaciens* pathovars represent genetically well-defined evolutionary lineages, isolated by specialisation on different plant hosts. These findings are further supported by the discovery of unique DNA sequences that can be employed for the specific detection of *C.f.* pv. *flaccumfaciens* strains and no other *C. flaccumfaciens* pathovars (Guimarães et al., 2001; Tegli et al., 2002). According to Dye et al. (1980), a pathovar is a strain or a set of strains with the same or similar characteristics that can be differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts. Although *C. flaccumfaciens* strains have been classified on the basis of this definition, the data provided by PFGE, in part supported by the analysis of the genome by AFLP and by rep-PCR, shows that variability can also be detected at the molecular level between the pathovars of this species raising the possibility that these methods can be used as alternatives to host testing.

References

Blears MJ, De Grandis SA, Lee H and Trevors JT (1998) Amplified fragment length polymorphism (AFLP): A review of the procedure and its applications. Journal of Industry and Biotechnology 21: 99–114

Bradbury JF (1986) Guide to Plant Pathogenic Bacteria, CAB International Mycological Institute, Kew, Surrey, UK

Carlson RR and Vidaver AK (1982) Taxonomy of *Corynebacterium* plant pathogens, including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. International Journal of Systematic Bacteriology 32: 315–326

Chen YF, Guo JH and Fang ZD (2000) A new pathovar of *Curtobacterium flaccumfaciens* on malabar spinach. Acta Phytopathologica Sinica 30: 171–175

Correia A, Martin JF and Castro JM (1994) Pulsed-field gel electrophoresis analysis of the genome of amino acid producing coryneform bacteria: Chromosome sizes and diversity of restriction patterns. Microbiology 140: 2841–2847

Davis MJ (1986) Taxonomy of plant-pathogenic coryneform bacteria. Annual Review of Phytopathology 24: 115–140

de Boer P, Dum B, Rigter A, van der Plas J, Jacobs-Reitsma WF and Wagenaar JA (2000) Computer-assisted analysis and epidemiological value for genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. Journal of Clinical Microbiology 38: 1940–1946

de Brujin FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerse chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Applied and Environmental Microbiology 58: 2180–2187

Dice LR (1945) Measures of the amount of ecologic association between species. Ecology 26: 297–302

Dye DW, Bradbury JF, Goto M, Hayward AC, Lelliott RA and Schroth MN (1980) International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Review of Plant Pathology 59: 153–168

Dye DW and Kemp WJ (1977) A taxonomic study of plant pathogenic *Corynebacterium* species. New Zealand Journal of Agricultural Research 20: 563–582

Felske A, Vancanneyt M, Kersters K and Akkermans ADL (1999) Application of temperature-gradient gel electrophoresis in taxonomy of coryneform bacteria. International Journal of Systematic Bacteriology 49: 113–121

Guimarães PM, Palmano S, Smith JJ, de SA, MFG and Saddler GS (2001) Development of a PCR test for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. Antonie van Leeuwenhoek 80: 1–10

Graves LM and Swaminathan B (1993) Universal bacterial DNA isolation procedure. In: Persing DH, Smith TF, Tenover FC and White TJ (eds) Diagnostic Molecular Microbiology: Principles and Applications (pp 617–621) American Society for Microbiology, Washington, DC

Hanninen ML, Perko-Makela P, Rautelin H, Duim B and Wagenaar JA (2001) Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electrophoresis genotypes studied by amplified fragment length polymorphism analysis, ribotyping and serotyping. Applied and Environmental Microbiology 67: 1581–1586

Harris-Baldwin A and Gudmestad N (1996) Identification of phytopathogenic coryneform bacteria using the Biolog automated microbial identification system. Plant Disease 80: 874–878

Henningson PJ and Gudmestad NC (1991) Fatty acid analysis of phytopathogenic coryneform bacteria. Journal of General Microbiology 137: 427–440

Jensen MA, Webster JA and Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction–amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology 59: 945–952

Keddie RM and Cure GL (1977) The cell wall composition and distribution of free mycolic acids in named strains of coryneform bacteria and in isolates from various natural sources. Journal of Applied Bacteriology 42: 229–252

Klaassen CHW, Harens HA van and Horrevorts AM (2002) Molecular fingerprinting of *Clostridium difficile* isolates: Pulsed-field gel electrophoresis *versus* amplified fragment length polymorphism. Journal of Clinical Microbiology 40: 101–104

Loreti S, Gallelli A, Belisario A, Wajnberg E and Corazza L (2001) Investigation of genomic variability of *Xanthomonas arboricola* pv. *juglandis* by AFLP analysis. European Journal of Plant Pathology 107: 583–591

Louws FL, Bell J, Medina-Mora CM, Smart CD, Opgenorth D and Ishimaru CA (1998) rep-PCR mediated genomic fingerprinting: A rapid and effective method to identify *Clavibacter michiganensis*. Phytopathology 88: 862–868

Louws FJ, Fullbright DW, Stephens CT and de Brujin FJ (1995) Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 85: 528–536

Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P, Prudhomme M, Alloing G, Hakenbeck R, Morrison DA, Boulnois GJ and Claverys JP (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Research 20: 3479–3483

McDonald JG and Wong E (2000) High diversity in *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* characterized by serology and rep-PCR genomic fingerprinting. Canadian Journal of Plant Pathology 22: 1–6

Mueller UG, Lipari SE and Milgroom MG (1996) Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. Molecular Ecology 5: 119–122

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P and de Bruijn FJ (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. International Journal of Systematic and Evolutionary Microbiology 50: 665–677

Smith JJ, Offord LC, Holderness M and Saddler GS (1995) Genetic diversity of *Burkolderia solanacearum* (synonym *Pseudomonas solanacearum*) Race 3 in Kenya. Applied and Environmental Microbiology 61: 4263–4268

Smith NC, Hennessy J and Stead D (2001) Repetitive sequence-derived PCR profiling using the BOX-A1R primer for rapid identification of the plant pathogen *Clavibacter michiganensis* subspecies *sepedonicus*. European Journal of Plant Pathology 107: 739–748

Sneath PHA and Sokal RR (1973) Numerical Taxonomy: The Principles and Practice of Numerical Classification. WH Freeman, San Francisco, USA

Sokal RR and Rohlf FJ (1962) The comparison of dendrograms by objective methods. Taxon 11: 33–40

Tegli S, Sereni A and Surico G (2002) PCR-based assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in bean seeds. Letters in Applied Microbiology 35: 331–337

Thwaites R, Mansfield J, Eden-Green S and Seal S (1999) RAPD and rep PCR-based fingerprinting of vascular bacterial pathogens of *Musa* spp. Plant Pathology 48: 121–128

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407–4414

Young JM, Bradbury JF, Davis RE, Dickey RS, Ercolani GL, Hayward AC and Vidaver AK (1991) Nomenclatural revisions of plant pathogenic bacteria and list of names 1980–1988. Review of Plant Pathology 70: 211–221

Zhao YF, Wei YD, Gao CS, Zhao LZ, Huang GM and Huang QL (1997) Using the Biolog identification system for rapid identification of bean wilt bacterium (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*). Acta Phytopathologica Sinica 27: 139–144