Molecular Characterization of *Burkholderia cepacia* Complex Isolates Causing Bacterial Fruit Rot of Apricot

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The *Burkholderia cepacia* complex isolates causing bacterial fruit rot of apricot were characterized by species-specific PCR tests, *recA*-*Hae*III restriction fragment length polymorphism (RFLP) assays, rep-PCR genomic fingerprinting, *recA* gene sequencing, and multilocus sequence typing (MLST) analysis. Results indicated that the isolates Bca 0901 and Bca 0902 gave positive amplifications with primers specific for *B. vietnamiensis* while the two bacterial isolates showed different *recA*-RFLP and rep-PCR profiles from those of *B. vietnamiensis* strains. In addition, the two bacterial isolates had a higher proteolytic activity compared with that of the non-pathogenic *B. vietnamiensis* strains while no *cblA* and *esmR* marker genes were detected for the two bacterial isolates and *B. vietnamiensis* strains. The two bacterial isolates were identified as *Burkholderia seminalis* based on *recA* gene sequence analysis and MLST analysis. Overall, this is the first characterization of *B. seminalis* that cause bacterial fruit rot of apricot.

Keywords : apricot fruit rot, *Burkholderia cepacia* complex, *Burkholderia seminalis*, *Burkholderia vietnamiensis*, identification

Bacterial fruit rot of apricot (*Prunus armeniaca*) (BFRA) has been first reported in China in 2009 and ascribed to *Burkholderia cepacia* based on carbon source utilization profile, fatty acid methyl esters and analysis of 16S rRNA gene sequence (Fang et al., 2009). However, *B. cepacia* has been regarded as a genetically distinct but phenotypically

similar bacteria group referring to *Burkholderia cepacia* complex (Bcc) (Fang et al., 2007, 2010; Mahenthiralingam et al., 2008; Zhang and Xie, 2007). Therefore, it is very necessary to determine the precise taxonomic position of the BFRA isolates in the Bcc by a variety of phenotypic and molecular analyses.

The Bcc currently comprises *B. cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia* and *Burkholderia ubonensis* (Coenye and Vandamme, 2003; Mahenthiralingam et al., 2008; Vermis et al., 2002). In addition, Vanlaere et al. (2008, 2009) recently proposed seven new species, namely *Burkholderia latens*, *Burkholderia diffusa*, *Burkholderia arboris*, *Burkholderia seminalis*, *Burkholderia metallica*, *Burkholderia contaminans* and *Burkholderia lata*. However, it is still unclear whether the BFRA isolates belong to one of these species or may represent a new and separate species of the Bcc.

The objective of this study was to identify and characterize the BFRA isolates by a combination of species-specific PCR tests, *recA*-*Hae*III restriction fragment length polymorphism (RFLP) assays, rep-PCR genomic fingerprinting, *recA* gene sequencing, and multilocus sequence typing (MLST) analysis as well as proteolytic activity. In addition, the cable pilin gene (*cblA*) and the *B. cepacia* epidemic strain marker (*esmR*) were detected among the BFRA isolates for the two genetic markers have been associated to the high transmissibility and virulence of some Bcc strains.

Materials and Methods

Bacterial strains and culture conditions. Two BFRA strains Bca 0901 and Bca 0902 were isolated from the fruit of apricot in our previous study (Fang et al., 2009). The reference strain LMG 1222^T of *B. cepacia* was provided by

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the Belgian Co-ordinated Collections of Microorganisms, BCCM, Gent, Belgium. In addition, the Bcc strains representative of different species were included in this study, which were isolated from the rhizosphere of rice (Zhang and Xie, 2007) and cystic fibrosis patients in China (Fang et al., 2010). All strains were maintained on nutrient agar medium (5 g of peptone, 1 g of beef extract, 2 g of yeast extract, 5 g of sodium chloride, and 15 g of agar per liter, PH 7.0). The incubation temperature for growth of bacterial strains was 30°C.

Species-specific PCR analysis. Total DNAs of bacterial isolates were extracted according to the method of Li et al. (2006, 2008). The putative species status of the BFRA isolates were determined by species-specific PCR tests, according to the procedures described previously (Mahenthiralingam et al., 2000). PCR reactions were performed with a Programmable Temperature Cycler (PTC-200, MJ Research, USA).

RecA-based RFLP analysis. Amplification of the *recA* gene was carried out by PCR as described by Zhang and Xie (2007) using the specific primers for Bcc, BCR1 and BCR2. *RecA*-RFLP was performed according to the method described previously (Lee and Chan, 2007; Mahenthiralingam et al., 2000). After amplification, 8 µl of amplified product was digested by 1 U of *Hae*III (Fermentas, USA) in a total volume of 15 µl and incubated at 37°C for 3 h. The RFLP products were separated by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. A 100-bp ladder (Fermentas, USA) was used as a molecular size marker. The RFLP patterns were manually analyzed and compared to those shown by Bcc reference strains (Mahenthiralingam et al., 2000).

Rep-PCR genomic fingerprinting. The rep-PCR of bacterial isolates and horizontal agarose gel electrophoresis of the PCR products was performed as described by Gevers et al. (2001) and Sahin et al. (2003). The rep-PCR oligonucleotide primers evaluated in this study were REP1R-I (5'-IIICGICGICATCIGGC-3') and REP2-I (5'-IIICGNC-GNCATCNGGC-3'), ERIC 1R (5'-ATGTAAGCTCCTGG-GGATTCAC-3') and ERIC2 (5'-AAGTAAGTGA CTGG-GGTGAGCG-3'), BOXA1R (5'-CTACGGCAAGGCGA-CGCTGACG-3'), and (GTG)₅ (5'-GTGGTGGTGGTGGTG-3') (Louws et al., 1995; Versalovic et al., 1994). PCR amplification was performed with a Peltier thermal cycler PTC-200 (MJ Research, Watertown, MA, USA) by using the following cycles: 1 initial cycle at 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 44, 52, 53, or 40°C for 1 min with REP, ERIC, BOX, and (GTG)₅ primers, respectively, and extension at 65°C for 8

min with a single final extension cycle at 65°C for 15 min and a final soak at 4°C. Analysis of the rep-PCR fingerprint profiles were done using the simple matching similarity coefficient and unweighted pair group with arithmetic averages using NTSYS-pc version 2.02e (Nazari et al., 2007; Versalovic et al., 1994).

DNA sequencing and phylogenetic analysis. PCR amplified product of the *recA* genes of the BFRA isolates were purified from the gel using AxyPrep DNA Gel Extraction Kit (Axygen BioSciences, USA) and cloned into pGEM-T Easy vector (Promega Co., Madison, WI, USA) according to the supplier's instructions and transferred into *Escherichia coli* (DH5α) cells. Clones with the correct insert were sequenced as described by Lee et al. (2009). Raw sequences were assembled using the SeqMan program and analyzed with the MegAlign programs (DNASTAR Inc., USA) as described by Jeon et al. (2006, 2008). Sequence identity was confirmed by analysis using the basic local alignment sequence tool (BLAST) at the National Center for Biotechnology Information (NCBI, Bethesda, Md.).

Phylogenetic analysis was performed on the *recA* sequence of the two BFRA isolates and 38 previously published Bcc *recA* gene (Baldwin et al., 2005; Coenye and Vandamme, 2003; Mahenthiralingam et al., 2000; Payne et al., 2005; Vandamme et al., 2002; Zhang and Xie, 2007). Nucleotides of the *recA* gene were aligned using CLUSTAL W. Phylogenetic and molecular evolutionary analyses were conducted using the genetic distance-based neighbor-joining algorithms within MEGA version 4.0 (<http://www.megasoftware.net/>). Bootstrap analysis for 1000 replicates was performed to estimate the confidence of tree topology. *Pseudomonas aeruginosa* was used as the outgroup.

MLST analysis. Seven genetic loci (*atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC* and *trpB*) of the BFRA isolates were amplified and sequenced by primers described previously (Baldwin et al., 2005). The forward and reverse sequences of a given locus were aligned, trimmed to the desired allele length using SeqMan II (DNA Star software) and compared with existing alleles in the Bcc MLST database at <http://pubmlst.org/bcc> (Jolley et al., 2004). Alleles were assigned arbitrary numbers for each locus and then the allelic profiles were compared using the MLST database in order to assign each isolate allelic profile with a sequence type (ST), or obtained new arbitrary numbers and new STs by depositing the new allele-profiles and isolate-profiles to MLST database. The different ST is an indicative of each genetically distinct strain (Baldwin et al., 2005).

A phylogenetic tree of concatenated sequences (2773 bp), including fragments of those seven genes from each isolate was constructed based on the neighbour-joining

method using MEGA software package version 4.0 (Baldwin et al., 2005; Kumar et al., 2004). The significance of branching within the trees was evaluated by bootstrap analysis of 1000 computer-generated trees. *Burkholderia pseudomallei* was used as the outgroup. This publication made use of the Bcc MLST website (<http://pubmlst.org/bcc/>) developed by Keith Jolley and sited at the University of Oxford (Jolley et al., 2004).

Detection of putative transmissibility markers. The detection of the two putative transmissibility markers (*cblA* and *esmR*) on the genome of the Bcc isolates was carried out by PCR as described previously (Clode et al., 2000; Mahenthiralingam et al., 1997). The 1.4-kb *esmR* gene was amplified with primers BCESM1/BCESM2, while the 664-bp *cblA* gene was amplified using primers CBL1/CBL2. Amplification reaction were carried out using Programmable Temperature Cycler for 25 cycles of 15 s at 95°C, 1 min at 63°C for detection of *esmR* or at 55°C for detection of *cblA*, 5 min at 70°C and then a final extension of 10 min at 70°C was applied to all thermal cycles.

Protease assays. Protease production of the Bcc isolates was performed on brain heart infusion (BHI) milk medium as described by Sokol et al. (1979) with minor modification. BHI milk medium was prepared by dissolving 18.5 g of BHI broth (Difco, USA) and 7.5 g agar (Difco, USA) in 400 ml of water. One hundred milliliter of 15% (wt/vol) solution of skim milk (Guangming, Heilongjiang, China) was prepared, and the solutions were autoclaved separately. These two sterile solutions were mixed at 60°C. A 10 ml volume was dispensed into Petri plates. The bacterial isolates were inoculated on BHI agar with a sterile toothpick. Protease activity was determined by measuring the dia-

meter (mm) of zone of clearing around the site of inoculation after incubating the plates for 24 h and 48 h at 30°C. Each treatment had four replicates, and the experiment was performed twice.

Results and Discussion

Species-specific analysis. Bacterial isolates were confirmed as Bcc by PCR amplification of the *recA* gene with specific primers for Bcc, BCR1 and BCR2 while an amplicon of about 1,041 bp was obtained from the two BFRA isolates. Species-specific PCR tests indicated that the two BFRA isolates and the reference strains of *B. vietnamiensis* gave positive amplifications with primers specific for *B. vietnamiensis* (Table 1). However, amplification of the two BFRA isolates with primers specific for *B. anthina* produced two amplicons of about 500 bp and 1000 bp (Table 1).

The results of this study support the general conclusion that the *recA* gene has proven very useful in discriminating Bcc from closely related species (Mahenthiralingam et al., 2000). Compared to the 16S rRNA gene, *recA* (RecA is a protein essential for repair and recombination of DNA) has been shown to be diploid, with a single copy of the gene residing on each of the two large chromosomes present in Bcc strains. If both copies of *recA* are identical, they may form a more robust platform upon which to base diagnostic PCR probes than the multicopy rRNA genes (Mahenthiralingam et al., 2000). However, this result is in contrast with the result of Mahenthiralingam et al. (2000), who found that species-specific PCR were able to distinguish the former nine species within Bcc. Therefore, it could be suggested that the BFRA isolates did not belong to one of these species.

Table 1. Species-specific PCR analysis of the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot

Species-specific primers	The apricot isolates		<i>B. vietnamiensis</i> strains ^a				
	Bca 0901	Bca 0902	419	430	440	S13	S23
<i>Burkholderia cepacia</i>	–	–	–	–	–	–	–
<i>B. multivorans</i>	–	–	–	–	–	–	–
<i>B. cenocepacia</i> IIIA	–	–	–	–	–	–	–
<i>B. cenocepacia</i> IIIB	–	–	–	–	–	–	–
<i>B. stabilis</i>	–	–	–	–	–	–	–
<i>B. vietnamiensis</i>	+	+	+	+	+	+	+
<i>B. dolosa</i>	–	–	–	–	–	–	–
<i>B. ambifaria</i>	–	–	–	–	–	–	–
<i>B. anthina</i>	+ ^b	+ ^b	–	–	–	–	–

^a The data of the reference strains are presented only for *B. vietnamiensis* because the isolates Bca 0901 and Bca 0902 gave positive amplifications with primers specific for *B. vietnamiensis*.

^b An amplicon of about 500 bp specific to *B. anthina* was obtained from the isolates Bca 0901 and Bca 0902 while another amplicon of about 1000 bp was also obtained.

Fig. 1. *RecA*-*Hae*III RFLP assays of the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot and the five reference strains of *B. vietnamiensis* with the enzyme *Hae*III. The DNA molecular size standard is in lane M. The Bcc isolates in each lane are as follows (second lane; left to right): Bca 0901; Bca 0902; *B. vietnamiensis* strains S13, S23, 419, 430, and 440. The alphabetical *Hae*III RFLP type is shown above each lane.

***RecA*-based RFLP.** Digestion with *Hae*III of *recA* gene of the two BFRA isolates resulted in a restriction pattern H, while digestion with *Hae*III of *recA* gene of the reference strains of *B. vietnamiensis* resulted in restriction patterns A and B (Fig. 1). This result is consistent with the result of Vandamme et al. (2002), who revealed that *recA*-*Hae*III RFLP types A and B represent *B. vietnamiensis*. However, it is well known that restriction pattern H was often observed for *B. cenocepacia* IIIB, *B. diffusa*, *B. arboris* and *B. seminalis* strains (Mahenthiralingam et al., 2000; Vanlaere et al., 2008), while these closely related species were able to be differentiated by comparing the sequences of the *recA* gene. The result of *recA*-RFLP is contrary with the result of *recA*-based species-specific PCR, which may be attributed to the complexity of the two BFRA isolates.

Rep-PCR genomic fingerprinting. Patterns of PCR products visualized over UV light by ethidium staining for BOX, ERIC, (GTG)₅, and REP primers are shown in Fig. 2a, 2b, 2c and 2d, respectively. The use of BOX and ERIC primers resulted in a banding pattern containing approximately 5–7 and 7–13 visualized PCR products, respectively, while the (GTG)₅ and REP primer generated fingerprints containing approximately 4–6 and 2–7 visualized PCR products, respectively. The dendrogram of combined data produced using BOX, ERIC, (GTG)₅, and REP primers is shown in Fig. 3. Cluster analysis of data resulting from profiles generated by rep-PCR revealed that the two BFRA isolates and the five reference strains of *B. vietnamiensis*

Fig. 2. Rep-PCR fingerprinting assays of the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot and the five reference strains of *B. vietnamiensis*. (A) BOX-PCR; (B) ERIC-PCR; (C) (GTG)₅-PCR; (D) REP-PCR. The DNA molecular size standard is in lane M. The Bcc isolates in each lane are as follows (second lane; left to right): Bca 0901; Bca 0902; *B. vietnamiensis* S13, S23, 419, 430, and 440.

Fig. 3. Dendrogram of genetic relatedness of the combined rep-PCR data inferred using primers BOX, ERIC, (GTG)₅, and REP and generated by the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot and the five reference strains of *B. vietnamiensis*. The scale indicates the degree of genetic relatedness between Bcc strains.

clustered into two groups and the average genetic similarity between the two groups was 0.42 (Fig. 3), which indicated that the BFRA isolates were genetically different from *B. vietnamiensis* strains, although they both gave positive amplifications with primers specific for *B. vietnamiensis*. In addition, more variation was found within *B. vietnamiensis* strains (average similarity coefficient 0.71) than in the two BFRA isolates (0.90).

DNA sequencing and phylogenetic analysis. To defini-

Fig. 4. Phylogenetic tree derived from the *recA* gene sequence analysis on reference strains of each *Burkholderia cepacia* complex species and the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot. The tree was generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Bootstrap analyses (1000 replicates) for node values from 50% are indicated. *Pseudomonas aeruginosa* was used as the outgroup. Bar, 1% sequence dissimilarity.

tively assess the species status of the two BFRA isolates, the complete *recA* gene sequences were determined and aligned to other known Bcc sequences deposited in Gen-

Fig. 5. Phylogenetic tree of concatenated nucleotide sequences from seven loci (*atpD*, *gltD*, *gyrB*, *recA*, *lepA*, *phaC* and *trpB*) of Bcc bacteria and the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot. The tree was generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Bootstrap analyses (1000 replicates) for node values from 50% are indicated. *Burkholderia pseudomallei* ATCC 23343^T was used as the outgroup. Bar, 1% sequence dissimilarity.

Bank. All sequences obtained were more than 96% similar to those of other members of the Bcc. The phylogenetic analysis revealed that the BFRA isolates and strains of *B. seminalis* clustered within a group and well separated from other species of Bcc (Fig. 4). It is therefore considered that the BFRA isolates should be identified as *B. seminalis*.

The result of phylogenetic analysis based on the *recA* gene sequence explained the differences in *recA*-RFLP and rep-PCR profiles between the two BFRA isolates and the *B. vietnamiensis* strains. However, species-specific primers were unavailable for the new proposed Bcc species including *B. seminalis*. Surprisingly, the BFRA isolates were unable to be differentiated from the strains of *B. vietnamiensis* based on the available species-specific primers,

indicating the complexity of the Bcc isolates.

MLST analysis. MLST analysis showed that the BFRA isolates Bca 0901 and Bca 0902 had a unique sequence type ST-590, which was novel when compared with existing STs in the MLST database. Concatenated nucleotide sequences (2,773 bp) for 141 Bcc STs to assess the relationships between the two BFRA isolates and different species of Bcc were analyzed using a neighbor joining tree (Fig. 5). Comparisons of the topology of neighbor-joining trees for the nucleotide sequence of the novel ST-590 clustered closely with STs belonged to *B. seminalis* (ST-260, -311, -343, -347, -383, -473, -508, -572) with 100% bootstrap values.

The MLST results were consistent with the result of phylogenetic analysis of the *recA* gene sequence, indicating that the two BFRA isolates belong to *B. seminalis*. Many researches have shown that MLST is a simple sequencing tool that uncovers allelic variants in conserved genes for the purpose of characterizing, typing, and classifying members of bacterial populations (Curran et al., 2004; Godoy et al., 2003). In agreement with the result of Vanlaere et al. (2008), this present study demonstrated that the MLST method was able to clearly differentiate all existing species in the closely related Bcc. In addition, the two BFRA isolates could be effectively identified by this simple, widely used technique that is directly comparable via the Internet.

Transmissibility marker genes. Pathogenic Bcc strains can sometimes be distinguished from nonpathogenic strains by examining the existence of transmissibility markers (Parke and Gurian-Sherman, 2001). Therefore, two transmissibility factors associated with the highly transmissible epidemic strains of *B. cenocepacia* were analyzed by PCR amplification. However, no *cblA* and *esmR* marker genes were detected from the two BFRA isolates and *B. cepacia* LMG 1222^T as well as the five reference strains of *B. vietnamiensis* (data not shown).

Protease assay. The zone diameter of hydrolysis around colonies of the BFRA isolates Bca 0901 and Bca 0902 were 1.5 mm and 0.7 mm, respectively, while the zone diameter of hydrolysis around colonies of *B. cepacia* LMG 1222^T was 0.5 mm on the BHI milk plates after 24 h incubation (Table 2). However, no visible zone of hydrolysis was observed around colonies of the five reference strains of *B. vietnamiensis*. The zone diameter of hydrolysis around colonies of the BFRA isolates Bca 0901 and Bca 0902 were 3.3 mm and 3.0 mm, respectively, while the zone diameter of hydrolysis around colonies of *B. cepacia* LMG 1222^T was 2.3 mm after 48 h incubation (Table 2). In addition, the

Table 2. Proteolytic activity of the isolates Bca 0901 and Bca 0902 causing bacterial fruit rot of apricot by inoculating them on brain heart infusion milk medium after inoculation of 24 h and 48 h

Bcc strains	Zones of clearing (mm) ^a after incubation of	
	24 h	48 h
Bca 0901	1.5±0.29	3.3±0.33
Bca 0902	0.7±0.33	3.0±0.00
<i>B. cepacia</i> LMG 1222 ^T	0.5±0.50	2.3±0.33
<i>B. vietnamiensis</i> 419	0.0±0.00	0.2±0.17
<i>B. vietnamiensis</i> 430	0.0±0.00	0.2±0.17
<i>B. vietnamiensis</i> 440	0.0±0.00	0.0±0.00
<i>B. vietnamiensis</i> S13	0.0±0.00	0.0±0.00
<i>B. vietnamiensis</i> S23	0.0±0.00	0.0±0.00

^a Zones of clearing were measured from edge of colony to edge of clearing.

zone diameter of hydrolysis around colonies of *B. vietnamiensis* strain 419 and 430 were 0.2 mm, while no visible zone of hydrolysis was observed around colonies of the other *B. vietnamiensis* strains.

In agreement with this result, Gingués et al. (2005) found that *B. cepacia* LMG 1222^T, an onion pathogen, shows proteolytic activity. In addition, Ludovic et al. (2007) revealed that a majority of Bcc isolates produce extracellular proteases. Interestingly, strains deficient in protease production are less virulent than the parental *B. pseudomallei* strain in diabetes-induced Sprague-Dawley rats, a lung infection model (Sexton et al., 1994). In this study, the plant pathogenic bacteria including the BFRA isolates and *B. cepacia* LMG 1222^T showed a higher proteolytic activity compared with that of the non-pathogenic *B. vietnamiensis* strains, which is consistent with the result of McKeivitt et al. (1989), who found that protease can be considered to be one of the virulence determinants of Bcc species.

In summary, our results clearly demonstrated that the BFRA isolates Bca 0901 and Bca 0902 had strong proteolytic activity and were identified as *B. seminalis* based on *recA*-RFLP and rep-PCR profiles as well as *recA* gene sequence analysis and MLST analysis. However, the two BFRA isolates were unable to be differentiated from the strains of *B. vietnamiensis* based on species-specific primers. To date, no *B. seminalis* strains have been reported as plant pathogen. This is the first characterization of *B. seminalis* that cause bacterial fruit rot of apricot.

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