Bacterial Brown Leaf Spot of Citrus, a New Disease Caused by *Burkholderia andropogonis*

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ABSTRACT

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A new bacterial disease of citrus was recently identified in Florida and is here named bacterial brown leaf spot (BBLS) of citrus. BBLS-infected citrus leaves from the field displayed circular, brownish, flat lesions with slightly raised and water-soaked margins surrounded by a chlorotic halo. Based on Biolog carbon source metabolic "fingerprinting", fatty acid analysis, and sequence analysis of partial 16S rDNA, *gyrB*, and *rpoD* genes, the causal agent of the disease was identified as *Burkholderia andropogonis*. Pathogenicity of these *B. andropogonis* isolates taken from multiple citrus leaves with BBLS was tested by various inoculation methods on three species of citrus as well as on carnation, corn, and sorghum. All isolates infected carnation, corn, and sorghum with varying degrees of pathogenicity. Variation among citrus isolates in pathogenicity was also observed in high titer (10^8 CFU/ml) inoculations of citrus leaves, ranging from a hypersensitive-like response to canker-like lesions. When the inoculum concentration was low (10^6 CFU/ml), only necrotic spots or small lesions slowly developed with all strains. Growth of *B. andropogonis* in citrus was relatively slow, tissue wounding appeared necessary for symptom appearance with many isolates, and field samples were recovered only after severe storms, indicating that this wide-host-range bacterium is a weak, opportunistic pathogen of citrus.

Burkholderia andropogonis was first described as the causal agent of stripe disease of sorghum and leaf spot of velvet bean in 1911 (20); at that time, it was classified as *Pseudomonas andropogonis*. *Pseudomonas woodsii* was also described, in the same publication, as an important pathogen of carnation. These two pathogens were reclassified to the genus *Burkholderia* based on DNA-rRNA hybridizations (13) and were shown to be synonymous based on phenotypic, genotypic, and chemotaxonomic evidence (9,13). Symptoms of *B. andropogonis*-infected leaves include brown, water-soaked, circular lesions with chlorotic halos. These lesions can coalesce, resulting in severe blight or leaf drop. When infected leaves are dissected in water, a stream of bacterial ooze is observed. *B. andropogonis* is now known to cause leaf spot, streaks, and

stripe on a wide variety of host plants covering 52 species from 15 families of unrelated monocotyledons and dicotyledons, including ornamentals such as white clover, carnation (26), and bougainvillea (29), and economically important crops such as corn, coffee, and chick pea (15). Recent reports show *B. andropogonis* to be a causal agent of diseases of jojoba (10), orchids (23), and golden cane palms (32). It can also survive on weeds such as Johnsongrass and sudangrass (8).

Since 2002, on both residential and commercial citrus plants, citrus leaves with flat, circular, brownish lesions, with slightly raised and water-soaked margins, surrounded by a chlorotic halo have been observed by citrus canker inspectors with the Division of Plant Industry, Florida Department of Agriculture and Consumer Services (DPI-FDAC) (21). The disease was observed only after storms that caused a high degree of damage and wounding to citrus trees. The flat lesions with slightly raised margins appeared somewhat similar to citrus canker (*Xanthomonas citri* pv. *citri*) and were therefore sent to the DPI-FDAC laboratory for further analysis and identification. However, the lesions were different from those of citrus canker disease, and were confirmed negative for *X. citri* by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Under the microscope, a bacterial stream was consistently observed from

leaf tissue slices cut from these lesions, and Gram-negative bacteria producing whitish colonies were consistently isolated on nutrient agar plates. All isolates, when inoculated into tomato and pepper, caused hypersensitive reactions (HR), indicating they were likely phytopathogenic. Further testing was conducted to confirm and to characterize the causal agent of what appeared to be a new citrus bacterial disease.

In this paper, we describe bacterial brown leaf spot (BBLS), a new, opportunistic bacterial disease of citrus in Florida, and identify the causal agent to be *B. andropogonis*. Furthermore, we have developed a simple and rapid genetic typing method for differentiating *B. andropogonis* strains using a simple PCR test based on a single primer.

MATERIALS AND METHODS

Bacterial isolation and pathogenicity tests. Samples were collected from residential and commercial citrus as possible citrus canker lesions from eight counties (Fig. 1A, Table 1). Diseased tissues were surfaced-sterilized and crushed in 1 ml of sterile tap H₂O (dH₂O) in a test tube using a glass rod. Samples were spread on nutrient agar (NA) plates and incubated at 28°C for 3 days. Whitish colonies of bacteria producing a diffusible nonfluorescent pigment were consistently isolated. Isolates were purified from single colonies and tested by Gram staining. Isolates were stored at room temperature in sterile tap H₂O and at -80°C in fresh nutrient broth containing 15% glycerol. The pathogenicity of all isolates was first screened by infiltrating the bacterial suspension in sterile tap H₂O at 10^8 CFU/ml into leaves of tomato (*Lycopersicon esculentum* var. *esculentum*), pepper (*Capsicum* spp.), and tobacco (*Nicotiana tabacum*). Isolates producing an HR on tomato and pepper were considered pathogenic. A total of 10 isolates were recovered from infected citrus. For comparison, *B. andropogonis* was isolated from *Bougainvillea* sp. and from carnation (*Dianthus caryophyllus*). The *B. andropogonis* type strain (ATCC 23061) from sorghum (renamed 1976 and kindly provided by Jeffery Jones, University of Florida) was also included in many tests (Table 1).

All 12 isolates and the type strain were inoculated into three citrus species: grapefruit (*Citrus × paradisi*), key lime (*C. au-*

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rantiifolia), and sweet orange (*C. sinensis*). These plants were 2 years of age and had been produced and maintained in greenhouse conditions. Various inoculation methods were evaluated, including spraying with and without wounding, needle puncturing, and pressure infiltrating by tuberculin syringes into newly expanded leaves on new flushes of all three citrus species. The concentration of inocula ranged from 10^8 to 10^3 CFU/ml with one log intervals. The concentrations of 10^8 , 10^7 , and 10^6 CFU/ml were chosen for infiltration studies and for histology. Samples eliciting different symptoms from inoculated citrus were collected, and the inoculated bacteria were reisolated in order to verify that the elicited symptoms were consistent. Inoculation controls included a citrus canker strain (*Xanthomonas citri* pv. *citri*), a citrus bacterial spot strain (*Xanthomonas axonopodis* pv. *citrumelo*), and

sterile tap H_2O . All 10 isolates from citrus were also tested by needle infiltration for their pathogenicity on three known hosts of *B. andropogonis* (4,18): carnation, corn (*Zea mays*), and sorghum (*Sorghum vulgare*), as described by Cother et al. (10).

Histology of lesions. Isolates 6370 and 6367 were inoculated into newly expanded leaves of sweet orange and grapefruit, and samples were collected 2 weeks later at the inoculation site. Multiple samples of 2×0.5 cm leaf tissues containing both healthy and inoculated lesion tissue were sectioned using a razor blade. The sections were placed in vials containing formalin-acid-alcohol and fixed for 2 h under a 500 hPa vacuum in a tissue processor. The sections were dehydrated in a series of alcohol and Histo-Clear baths and then embedded in paraffin. The embedded leaf tissue was cut with a microtome to a thickness of 10 to 15 μ m, floated on warm H_2O , and mounted

on Poly-L-Lysine coated microscope slides. The mounted sections were placed on a slide warmer at 42°C for 12 h. The paraffin was removed from the tissue sections by placing the microscope slides in Histo-Clear 2 times for 3 min, then rehydrated in 95, 85, 70, 50, and 30% ETOH 2 times in each solution for 3 min each and a final hydration of 5 min in ddH₂O. The leaf sections were then stained with Safranin O for 15 min, and then washed with running dH₂O until clear. The samples were dehydrated again in a series of alcohol and Histo-Clear baths as described above. A cover glass was then mounted onto the tissue sections with paramount. The samples were examined under a microscope and photographed.

In vivo population dynamics. Potted greenhouse-grown grapefruit plants were pruned to produce uniformly aged shoots, and three young shoots were inoculated

Fig. 1. Field sample and phenotypic variations of citrus leaves inoculated with different isolates of *Burkholderia andropogonis*. **A**, Original field sample of bacterial brown leaf spot (BBLS) on grapefruit. **B**, Grapefruit leaf inoculated with isolate 6269 at concentration 10^7 CFU/ml using infiltration. B was photographed 2 weeks after inoculation with $\times 16$ close-up. **C**, Key lime leaf inoculated with isolate 6269 at concentration 10^7 CFU/ml using needle puncture, photographed 3 weeks after inoculation. **D**, Grapefruit leaf inoculated with isolate 6269 at concentration 10^7 CFU/ml using needle puncture, photographed 3 weeks after inoculation. **E**, Hypersensitive reaction (HR)-like and chlorosis response on grapefruit leaves inoculated with isolate 6177 at 10^8 CFU/ml (1 and 2) and 10^6 CFU/ml (3), respectively, photographed 4 days after inoculation. **F**, Canker-like lesion on grapefruit inoculated with isolate 6367 at concentration 10^8 CFU/ml (1) and 10^5 CFU/ml (2), respectively, and healthy control (3), photographed 2 weeks after inoculation.

per plant. Single colonies from isolates 6269 and 6369 were transferred separately to nutrient broth and grown to log-phase. The cultures were then pelleted by centrifugation, resuspended in sterile tap water, and adjusted to a 10^4 CFU/ml culture. The bacterial suspensions were infiltrated into 15 individual leaves on the young grapefruit shoots, using a 1-ml tuberculin syringe and 25-gauge needle. Inoculated plants were kept in a quarantine greenhouse at 20 to 30°C. Infiltrated leaves were sampled at 0, 2, 4, 8, and 12 days after infiltration. At each sample period, two leaf disks (about 1 cm² total) from infiltrated tissue were triturated in sterile tissue grind-

ers containing 1 ml of sterile tap water. The suspension was serially diluted 10-fold, and 100 µl per dilution were spread onto nutrient agar plates. Three infiltrated leaves were used as replications from each time interval. The colonies on each plate were counted after incubation at 28°C for 72 h. The experiment was conducted twice.

Carbon utilization and fatty acid analysis. Pure cultures of isolates 6269 and 6369 were grown and tested for utilization of the 95 carbon sources available on GN Microplate (Biolog Inc., Hayward, CA) as previously described (3). The carbon utilization patterns were read with a microplate reader and analyzed by a

cluster analysis program provided by Biolog.

Fatty acid profiles were generated for six selected isolates. The bacterial cultures were grown on trypticase soy broth agar (BBL Laboratories, Cockeysville, MD) at 28°C for 24 h. Whole-cell fatty acid methyl esters (FAME) were extracted and characterized at the Bacterial Identification and Fatty Acid Analysis Laboratory, Plant Pathology Department, University of Florida, Gainesville. Profiles were pooled and subjected to the Principal Components Analysis using the Microbial Identification System (MIS) software to determine relationships among isolates. The fatty acid

Table 1. Bacterial isolates used in this study

Strain	Syn.	Species	Origin	Host
1894	XS2002-0001	<i>B. andropogonis</i>	Grove, Desoto County	<i>Citrus</i> sp.
6173	XC2004-00267	<i>B. andropogonis</i>	Grove, Polk County	<i>C. sinensis</i>
6177	XI2004-00506	<i>B. andropogonis</i>	Resident, Lee County	<i>Citrus</i> sp.
6268	XN2005-1111	<i>B. andropogonis</i>	Resident, Orange County	<i>C. sinensis</i>
6269	XI2004-856	<i>B. andropogonis</i>	Resident, Lee County	<i>Citrus</i> sp.
6367	XI2005-0014	<i>B. andropogonis</i>	Resident, Lee County	<i>Citrus</i> sp.
6368	XC2005-0161	<i>B. andropogonis</i>	Resident, Indian River County	<i>Citrus</i> × <i>paradisii</i>
6369	XC2005-0162	<i>B. andropogonis</i>	Resident, Indian River County	<i>C. sinensis</i>
6370	X2005-1008-2	<i>B. andropogonis</i>	Resident, Palm Beach County	<i>C. reticulata</i>
6758	XC2005-283	<i>B. andropogonis</i>	Resident, St Lucie County	<i>C. sinensis</i>
1068	P1998-2547	<i>B. andropogonis</i>	Nursery, Marion County	<i>Dianthus caryophyllus</i>
7665	P2006-15	<i>B. andropogonis</i>	Resident, Palm Beach County	<i>Bougainvillea</i> sp.
1976	...	<i>B. andropogonis</i>	ATCC 23061 ^a	<i>Sorghum vulgare</i>
D294	...	<i>B. glumae</i> ^b	Arkansas	Rice
D502	...	<i>B. glumae</i> ^b	Arkansas	Rice
DNA	...	<i>B. gladioli</i> ^b	...	...
GMI 1000	...	<i>Ralstonia solanacearum</i>	Guyana	Tomato
3213	...	<i>Xanthomonas citri</i> pv. <i>citri</i>	Florida	Citrus
1660	...	<i>X. citri</i> pv. <i>citri</i>	Florida	Citrus
C340	...	<i>X. citri</i> pv. <i>aurantifolia</i>	ATCC 51302	Citrus
1377	...	<i>X. axonopodis</i> pv. <i>citrumelo</i>	Florida	Citrus
DH 5α	...	<i>Escherichia coli</i>	New England Biolabs	...

^a Strain provided by Jeffrey B. Jones, University of Florida.

^b Strains and DNA provided by Ronald J. Saylor, University of Arkansas.

Fig. 2. Lesions induced by *Burkholderia andropogonis* isolate 6367 and thin-section microscopy of the infected tissue. **A**, Lesion on sweet orange. **B**, Thin section of a 2-week-old lesion on sweet orange. Note hypertrophy and absence of hyperplasia. **C**, Thin section of a healthy control leaf of sweet orange. **D**, Lesion on grapefruit. **E**, Thin section of a 2-week-old lesion on grapefruit. Note hypertrophy and absence of hyperplasia. **F**, Thin section of a healthy control leaf of grapefruit.

compositions of the isolates were also used to produce an unweighted pair-matching dendrogram based on cluster analysis techniques.

DNA isolation, PCR amplification, and sequence analysis of 16S rDNA, *gyrB*, and *rpoD*. Genomic DNA of all unknown isolates was extracted according to manufacturer's instructions (Genomic-Prep Kit, Amersham Pharmacia Biotech, Piscataway, NJ). PCR amplifications of partial 16S rDNA, *gyrB*, and *rpoD* were performed using primer pairs fD1 and rD1, LJ23f and LJ24r, and LJ25f and LJ26r, respectively (Table 2). The PCR reactions were performed using PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA) in a 25- μ l reaction consisting of 1 $\times$ buffer D (Epicentre Biotechnologies, Madison, WI) and 1 unit of *Taq* polymerase (New England BioLabs, Ipswich, MA) under the following conditions: 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension cycle of 10 min at 72°C. PCR products were purified with a PCR purification kit (QIAquick, Qiagen, Germantown, MD) and were cloned into the pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and identified by direct PCR amplification using M13 forward and reverse primers. The identified clones were grown in LB broth overnight, and DNA was isolated using a plasmid mini kit (Qiagen). DNA sequencing was performed with the BigDye Terminator Cycle Sequencing Kit v 3.1 and M13 universal sequencing primers on the AB 3730xl

DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were compared with previously identified sequences in the NCBI database using BLAST (2). The sequences of 16S rDNA obtained in this study were aligned with closely matched sequences from the NCBI database using the pileup function of the GCG Package (Accelrys Inc., San Diego, CA) and adjusted manually with ClustalX version 1.8 (24). Phylogenetic trees were generated with Treecon (27,28) using a neighbor-joining method. Bootstrap analysis was performed with 1,000 resamplings of the DNA sequences to estimate the confidence of the tree topology.

PCR-based genotyping and sequence analysis of the PCR products. The genetic diversity of citrus isolates was analyzed using a single primer, DG04, which targets an inverted terminal repeat sequence of *X. citri* pv. *citri* (11). The amplified DNA fragments generated from the PCR reaction were separated on a 1.0% agarose gel in TAE buffer, and visualized with ethidium bromide staining. PCR products from selected isolates were cloned into the pCRII-TOPO cloning vector and identified by direct PCR amplification using M13 forward and reverse primers. The identified clones were grown in LB broth overnight, and DNA was isolated using a plasmid mini kit (Qiagen). DNA sequencing of the clones was performed by the DNA Sequencing Core Laboratory, Interdisciplinary Center for Biotechnology Research at the University of Florida. Putative functional homologues of the obtained

sequences were identified by comparing with previously identified sequences in the NCBI database using BLAST.

RESULTS

Bacterial isolation and pathogenicity tests. Leaves exhibiting BBLS symptoms were recognized as distinctive from both citrus bacterial spot and Asiatic citrus canker disease (Fig. 1A). The predominant bacterial colonies appearing on nutrient agar plates from ground BBLS leaf samples were whitish and produced a diffusible nonfluorescent pigment. These colonies were consistently isolated from all diseased leaves exhibiting characteristic BBLS symptoms. A total of 10 isolates, one from each of the BBLS field samples of citrus (Table 1), were obtained. The bacteria from these colonies elicited an HR on both tomato and pepper, but not tobacco, when infiltrated into leaves at 10⁹ CFU/ml. All isolates were pathogenic, although in varying degrees, on corn, carnation, and sorghum, causing stripe symptoms on corn and sorghum, and blight symptoms on carnation.

On citrus, the isolates showed significant variation in their pathogenicity and their ability to elicit host responses when using infiltration inoculation (Figs. 1 and 2, Table 3). When a high concentration (10⁸ CFU/ml) of isolates 6177, 6758, and 1894 were infiltrated into newly expanded citrus leaves, an HR-like symptom was elicited (Fig. 1E). This HR-like symptom was readily apparent at approximately 48 h after inoculation, and abscission of inoculated leaves occurred 24 to 48 h later. In contrast, the HR elicited by *X. citri* pv. *citri* strain A^W and *X. citri* pv. *aurantifolia* strain C on grapefruit appeared at least 72 h after inoculation, and the rapid abscission of inoculated leaves was not observed (data not shown). When the inoculum was diluted 100-fold to 10⁶ CFU/ml, these isolates caused either chlorosis or no obvious symptoms (Fig. 1E). Isolates 6268, 6269, and 6370, when infiltrated at high

Table 2. Polymerase chain reaction (PCR) and sequencing primers used in this study

Primer	Target gene	Sequence (5'-3')	Reference
fD1	16S rDNA	AGAGTTTGATCCTGGCTCAG	30
rD1		AAGGAGGTGATCCAGCC	30
LJ23f	<i>gyrB</i>	GGGGGTGTCTTTCGTAAC	This study
LJ24r		ACACCAGCTTGCTTCGTCT	This study
LJ25f	<i>rpoD</i>	CGCCAAACGGATCGAGGAAGG	This study
LJ26r		TCACCCAGCGGCAACACGAC	This study
DG04	Invert repeats	AAT CCC TGC CGA CCC TC	11

Table 3. Pathogenicity tests on sweet orange, key lime, and grapefruit and selected known hosts of *Burkholderia andropogonis* following high level leaf tissue infiltration inoculation with bacterial brown leaf spot (BBLS) isolates and selected strains of *B. andropogonis*

Isolate/strain	Sweet orange	Key lime		Grapefruit		Carnation ^a	Corn ^a	Sorghum ^a
		Infiltration	Needle-puncture	Infiltration	Needle-puncture			
6173	Ch ^b	Ch++	+	Ch++	+	+++	+++	+
6177	HR+++	HR+++	±	HR+++	±	+	+	+
6268	Ch++	HR+++	++	C++	++	++	++	+++
6269	Ch++	C++	++	C++	++	+	++	+
6367	C+++	C++	++	C++	++	+++	+++	+++
6368	HR+++	HR+++	+	Ch++	+	+++	+++	+
6369	HR++	HR++	+	C++	+	++	++	++
6370	Ch++	HR++	++	C++	++	++	++	+
6758	HR+++	HR+++	+	HR+++	+	+++	++	+++
1894	HR+++	HR+++	+	HR+++	+	++	+++	+++
1068	HR+++	HR++	+	Ch++	+	+++	+++	+
1976	Ch++	Ch+	±	HR+++	±	+++	+++	+++
7665	HR+++	HR+++	±	Ch+	±	+++	+++	++

^a Symptoms scored after 2 weeks.

^b Ch = Chlorosis (after 2 weeks); HR = hypersensitive-like response (after 2 days); C = Canker-like lesions (after 2 weeks). +, ++, +++ represents apparent intensity of response.

levels, caused raised lesions similar to citrus canker disease after 1 to 2 weeks, but only on grapefruit; on sweet orange and key lime, only small, flat lesions with raised margins appeared after 2 weeks. Following low level infiltration, lesions elicited by these strains appeared after 1 month (i.e., small canker-like lesions on grapefruit and small flat lesions with raised margins on sweet orange and key lime). Isolate 6367 caused canker-like lesions (Fig. 2A and D) on all three species of citrus tested after 1 to 2 weeks following high level inoculations, and small canker-like lesions on all three species after 2 to 4 weeks at low level inoculations.

Spraying of a low concentration (10^6 CFU/ml) of any of these BBLS isolates on nonwounded citrus leaves resulted in no lesions. In contrast, all isolates at a low concentration caused lesions (Fig. 1C and D) on citrus with needle-puncture inoculation; however, the resulting lesions were of varied sizes and required longer than a month to develop. Wounding of citrus tissue therefore appeared to be critical for lesion development from low level inoculations.

Histology of *B. andropogonis* lesions. Isolates of *B. andropogonis* from BBLS citrus caused raised, necrotic, and canker-like lesions when the bacteria were inoculated into newly expanded citrus leaves. Thin-section microscopy of infected grapefruit and sweet orange tissues revealed an increase in the volume of mesophyll cells (i.e., hypertrophy) and the loss of free extracellular space in the apoplast in the raised lesion area (Fig. 2). In contrast to citrus canker disease, no hyperplasia was observed.

In vivo growth dynamics. Growth kinetic studies revealed the bacterium grew relatively slowly inside citrus leaf tissue. According to the growth curves produced for the two isolates, 6269 and 6369, the populations exhibited less than two-log growth over a 12-day period and achieved only a low overall titer in citrus, less than 10^4 CFU/cm² (Fig. 3).

Identification of the causal agents of BBLS. All 10 citrus BBLS isolates were Gram-negative. Isolates 6269 and 6369 were identified as *B. andropogonis* using carbon utilization profiles. Isolates 6268, 6269, 6367, 6368, 6369, and 6370 were identified as *B. andropogonis* using FAME analysis, and their index values varied from 0.692 to 0.934. The dendrogram produced based on fatty acid composition showed that these six isolates formed a cluster with *B. andropogonis*, separate from *B. glumae* and *B. gladioli*. They appear to be the same species (Fig. 4).

Uniform DNA fragments of ca. 1,500 bp were amplified by PCR using universal 16S rDNA primers; no polymorphism was observed following restriction enzyme digestion using *Hha*I and *Tag*I (data not shown). The 16S rDNA sequences from all isolates and the type strain were obtained.

Isolates 1068 (accession no. FJ387578), 1894 (FJ387579), 1976 (FJ387580), 6173 (FJ387581), 6177 (FJ595135), 6268 (FJ387582), 6269 (DQ786950), 6367 (FJ387583), 6368 (FJ387584), 6369 (DQ786951), 6370 (FJ387585), 6758 (FJ387586), 6893 (FJ387587), and 7665 (FJ387588) shared more than 99% identities with the previously reported *B. andropogonis* 16S rDNA gene sequences in the NCBI database. The phylogenetic placement and relationship of the representative BBLS isolates to other closely related plant pathogenic bacteria using 16S rDNA sequences is illustrated in Figure 5. The results of the 16S rDNA sequence analysis confirmed the identification of the citrus BBLS isolates as *B. andropogonis*.

In addition, uniform DNA fragments amplified by PCR using *gyrB* and *rpoD* were obtained, cloned, and sequenced. All sequences from *gyrB* and *rpoD* also shared over 99% identities with the previously reported *B. andropogonis gyrB* and *rpoD* sequences, respectively, in the NCBI database (Table 4). The results of the sequence analysis of these two house-keeping genes, *gyrB* and *rpoD*, further confirmed the identification of the citrus BBLS isolates as *B. andropogonis*. Primers designed for *gyrB* were specific for PCR detection of *B. andropogonis* among the tested bacterial samples (Fig. 6).

Genetic diversity of citrus isolates analysis by PCR. Differing patterns of PCR products were observed among *B.*

Fig. 3. Population dynamics of *Burkholderia andropogonis* isolates 6269 and 6369 in grapefruit (*Citrus × paradisi*). Data are means of three replications repeated twice ($n = 6$) $\pm$ standard error of the mean.

Fig. 4. Dendrogram showing the relationships of *Burkholderia andropogonis* strains isolated from citrus and other *Burkholderia* species based on the fatty acid analysis.

andropogonis citrus isolates using the inverted repeat sequence-based primer DG04. Four of the 10 citrus isolates examined (6268, 6269, 6367, and 6370) shared a similar pattern of two PCR products (Fig. 7). Sequence analysis of the DNA fragments of one of the four isolates, 6269, revealed the presence of a phage integrase-like protein composed of 261 amino acids (EU346201) in the large fragment, and presence of an ABC transporter-like protein composed of 131 amino acids

(EU346202) in the small fragment. Isolate 6177 displayed a unique PCR amplification pattern, and the amplicon was identified as a putative iron-sulfur-binding reductase-like protein (EU909698). The PCR products of primer DG04 revealed different genetic elements, indicating a degree of genetic diversity among the isolates.

DISCUSSION

This is the first report of bacterial brown leaf spot (BBLs) of citrus, a new and ap-

parently opportunistic bacterial disease of citrus. Typical symptoms of the disease are flat, circular, brownish lesions with slightly raised and water-soaked margins surrounded by a chlorotic halo. The occurrence of the disease in the field was consistently associated with storms that caused damage to citrus, indicating the bacteria infected citrus plants via wounds. By fulfilling Koch's postulates and discerning that the causal agent was indeed a bacterium, we eliminated other diseases of cit-

Fig. 5. Neighbor-joining phylogenetic placement of *Burkholderia andropogonis* and related plant pathogens. Analysis based on approximately 1,500-nt sequences from positions 27–1543 (based on *Escherichia coli* numbering) of 16S rDNA with *Agrobacterium tumefaciens* as the outgroup taxon. Scale bar represents 10% change. Numbers at nodes represent percentage of bootstrap resampling based on 1,000 replicates.

Table 4. Comparison of fatty acid analysis and *gyrB*, *rpoD* gene sequence analyses results for identification of *Burkholderia andropogonis* isolates

Isolate/strain	Biolog I.D.	Fatty acid sim index ^a	<i>gyrB</i> BLAST ^b			<i>rpoD</i> BLAST ^b		
			Score	E-value	Acc. num.	Score	E-value	Acc. num.
6173	N/A	N/A	1279	0.0	EU265649	1369	0.0	EU306162
6177	N/A	N/A	1279	0.0	EU265650	1369	0.0	EU306163
6268	N/A	0.739	1279	0.0	EU265651	1369	0.0	EU306164
6269	<i>B. andropogonis</i>	0.696	1279	0.0	EU265652	1369	0.0	EU265662
6367	N/A	0.692	1279	0.0	EU265653	1369	0.0	EU306165
6368	N/A	0.908	1279	0.0	EU265654	1369	0.0	EU306166
6369	<i>B. andropogonis</i>	0.934	1284	0.0	EU265655	1369	0.0	EU306167
6370	N/A	0.734	1279	0.0	EU265656	1369	0.0	EU306168
6758	N/A	N/A	1279	0.0	EU265657	1363	0.0	EU306169
1894	N/A	N/A	1279	0.0	EU265658	1369	0.0	EU306170
1068	N/A	N/A	1279	0.0	EU265659	1369	0.0	EU306171
1976	N/A	N/A	1279	0.0	EU265660	1369	0.0	EU306172
7665	N/A	N/A	1279	0.0	EU265661	1369	0.0	EU306173

^a A similarity index of fatty acid greater than 0.8 is considered an exact match.

^b All BLAST score values represent the highest match to *B. andropogonis gyrB* (GenBank accession no. AB190572) and *rpoD* (GenBank accession no. AB190669).

rus which display similar phenotypes. These included the fungal diseases: greasy spot (*Mycosphaerella citri*) (31), Phaenarumularia fruit and leaf spot (*Pseudocercospora angolensis*), and Alternaria brown spot (*Alternaria citri*) (7,25).

The bacteria only caused lesions when they were inoculated by needle puncture or infiltration but not by spraying without wounding, confirming the field observation that the bacteria infected plant tissues via wounds. A growth dynamics study revealed that the multiplication of BBLS isolates from citrus was slow, increasing less than two logs in 12 days (Fig. 3). When compared to a four-log increase in 8 days of *X. citri* pv. *citri* (21), this study indicates that the BBLS isolates are poor pathogens of citrus and supports the opportunistic nature of BBLS field infections. Based on the results of Biolog metabolic profiles, FAME analysis, and sequence analysis of 16S rDNA, *gyrB*, and *rpoD* genes, the causal agent of the bacterial brown leaf spot disease of citrus was identified as *Burkholderia andropogonis*. This eliminated other bacterial diseases that cause lesions: citrus bacterial spot (CBS; *X. axonopodis* pv. *citrumello*) and citrus canker (*X. citri*) (12,19). The necessity of wounding for infection by *B. andropogonis* has been observed in other host plants,

including *Limonium sinuatum*, *Bougainvillea spectabilis*, *Coffea arabica* (17), and carnation (6).

Unlike in *B. glumae* and *B. gladioli* (16), sequence analysis of the *gyrB* and *rpoD* genes did not differentiate among the BBLS isolates; all of them share more than 99% identities, even those that elicited different host responses. However, cellular fatty acid composition indices of the BBLS isolates ranged from 0.692 to 0.934. The same variation has also been reported in *B. andropogonis* strains isolated from jojoba, where the similarity index ranged from 0.342 to 0.829 (10). Furthermore, when using the DG04 primer, which was designed for genetic typing of *X. citri* (citrus canker) strains (11) based on the terminal inverted repeat sequences that flank the pathogenicity gene *pthA* (22), we obtained different PCR products from these isolates (Fig. 7). Four of the 11 isolates examined shared a similar pattern of PCR products using DG04, and these same isolates/strains were found to be more pathogenic to citrus.

B. andropogonis infects a wide variety of host plants and causes leaf spot, streaks, stripe, and blight on unrelated monocotyledons and dicotyledons (10,17,23,29,32). Leaf lesions on these plants vary in their color, including tan, purple, brown, and

black, with variations occurring not just between the hosts but among hosts. Symptoms on carob, *Ceratonia siliqua* L., have been described as dark tan to black lesions, irregular in shape, with a paler green margin (17). The symptom variation also includes the lesion size as in jojoba, *Simmondsia chinensis*, where lesions are described as ranging from "necrotic flecking" with water-soaked halos to larger "eyespot" (10). *B. andropogonis*, along with *B. glumae* and *B. plantarii*, produces non-host-specific toxins that induce chlorosis or necrosis of plant tissue (14). It is intriguing that when infiltrated at high concentrations, some of the BBLS isolates induced a rapid HR-like response in citrus. Unlike *X. citri* pv. *citri* A^W and *X. citri* pv. *aurantifolia* strain C (1), the HR-like responses induced by the BBLS isolates were more rapid and were not citrus host specific. Furthermore, no hypersensitive response and pathogenicity (*hrp*) genes, necessary to produce an HR, have been reported in *B. andropogonis*. On the other hand, the culture filtrates of these isolates from nutrient broth did not exhibit any toxic effects on citrus (data not shown). Whether the HR-like phenotype induced by the BBLS isolates is controlled by Type III effector genes, toxins, or some other mechanism entirely remains to be investigated.

Fig. 6. Polymerase chain reaction (PCR) test for the specificity of primer LJ23f/LJ24r designed from *Burkholderia andropogonis* *gyrB* gene. Lane M, 1-kb DNA ladder; lanes 1-13, *B. andropogonis* 6173, 6177, 6268, 6269, 6367, 6368, 6369, 6370, 6758, 1068, 1894, ATCC23061, 7665; lane 14, *Xanthomonas axonopodis* pv. *citri* 3213; lane 15, *X. axonopodis* pv. *citri* 1660; lane 16, *X. axonopodis* pv. *citrumelo* 1377; lanes 17-18, *Burkholderia glumae* D294, D502; lanes 19-20, *Burkholderia* sp.; lane 21, *Escherichia coli* DH5 α ; lane 22, *Ralstonia solanacearum* GMI 1000; lane 23, water control.

Fig. 7. Genetic diversity analysis of *Burkholderia andropogonis* isolates using polymerase chain reaction (PCR) amplification with DG04 primer. M, 1-kb DNA marker (Promega, Madison, WI). Lanes 1-11, *B. andropogonis* isolates 1068, 1976, 6173, 6177, 6268, 6269, 6367, 6368, 6369, 6370, and 7665, respectively.

BBLs was discovered because these isolates were submitted by state inspectors as citrus canker suspects. Thus, the lesions of BBLs obviously can be confused with those of citrus canker. Typical canker symptoms include raised, erumpent lesions with a water-soaked margin, and are histologically characterized by both hypertrophy and hyperplasia (5). BBLs elicits small lesions that are flat with slightly raised margins. Microscopically, *B. andropogonis* causes hypertrophy but does not induce hyperplasia. The causal agent of citrus bacterial spot, *X. axonopodis* pv. *citrumelo*, also causes flat, water-soaked leaf spots on juvenile citrus (12,22), but this nursery disease does not affect mature trees. Although there are some symptom similarities among these bacterial diseases, BBL lesions can be distinguished by a reddish appearance on grapefruit (Fig. 1A) and sweet orange. Since BBL lesions on citrus can be confused with atypical lesions of citrus canker, it is important for inspectors and growers to be informed regarding this new bacterial disease of citrus in order to limit confusion. Also due to the difficulty in differentiating BBLs from atypical citrus canker and bacterial spot by symptomology, serological and molecular tools are necessary to aid in diagnosis. The primers DG04 and those that target the *gyrB* and *rpoD* genes of *B. andropogonis* developed from this study may be used for future PCR detection and diagnosis.

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