***Burkholderia glumae* and *B. gladioli* Cause Bacterial Panicle Blight in Rice in the Southern United States**

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ABSTRACT

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Bacterial panicle blight (BPB) is among the three most limiting rice diseases in Louisiana and the southern United States. The identity and characterization of pathogens associated with this disease was unclear. This research details studies carried out on the pathogens causing BPB on rice in Louisiana and other rice producing southern states. Bacterial strains were isolated from BPB-infected sheath, panicle, or grain samples collected from rice fields in Louisiana, Arkansas, Texas, and Mississippi. In greenhouse inoculation tests, 292 of 364 strains were pathogenic on rice seedlings or panicles. Identification of strains in the pathogen complex by growth on S-PG medium, carbon source utilization profile (Biolog), cellular fatty acid analysis, and polymerase chain reaction (PCR) methods revealed that 76 and 5% of the strains were *Burkholderia glumae* and *B. gladioli*, respectively. The other strains have not been conclusively identified. Although strains of both species produced similar symptoms on rice, *B. glumae* strains were generally more aggressive and caused more severe symptoms on rice than *B. gladioli*. Virulent strains of both species produced toxoflavin in culture. The two species had similar growth responses to temperature, and optima ranged from 38 to 40°C for *B. glumae* and 35 to 37°C for *B. gladioli*. PCR was the most sensitive and accurate method tested for identifying the bacterial pathogens to the species level. The 16S rDNA gene and 16S-23S rDNA internal transcribed spacer (ITS) region sequences of the *B. glumae* and *B. gladioli* strains from rice showed more than 99% sequence homology with published sequences. A real-time PCR system was developed to detect and quantify this pathogen from infected seed lots. Our results clearly indicate that *B. glumae* and *B. gladioli* were the major pathogens causing BPB in the southern United States.

Rice production in the southern United States has a long history of loss to panicle blighting of unknown etiology (7). Epidemics of panicle blight occurred during 1995 and 1998, years of record high nighttime temperatures, with yield losses in some fields estimated to be as high as 40%. Significant losses were also experienced in Louisiana during 2000 (20,30). Earlier, panicle blighting was attributed to abiotic factors including high temperature, water stress, or toxic chemicals near the root zone (7), but in 1996–97, the bacterial plant pathogen *Burkholderia glumae* (formerly *Pseudomonas glumae*) was identified as a cause of panicle blighting in the southern United States (30). This bacte-

rium was first described in Japan as the cause of grain rotting and seedling blight on rice (6). The disease was later reported from other Asian countries and Latin America (2,4,10,40). *B. glumae* produces the phytotoxin toxoflavin, which is essential for its virulence. Strains that lack toxin production become avirulent (9,26). The symptoms of bacterial panicle blight (BPB) include seedling blight, sheath rot, and panicle blight with significant yield losses (2,27,34,39). Specific sheath symptoms include long, vertical grayish lesions surrounded by a dark reddish-brown margin (Fig. 1A). The disease is characterized as having upright, straw-colored panicles containing florets with a darker base and a reddish-brown line (margin of lesion) across the floret between the darker area and the straw-colored area, resulting in abortion of the kernel before it fills (Fig. 1B). Severely affected panicles remain upright, as the grain does not fill (Fig. 1C). The term “panicle blight” has been used in the United States for more than 50 years, and

BPB has been retained as the name for the disease in this country.

As no published reports were available for this disease in North America in 1996 and most of the commercially grown U.S. rice varieties were susceptible to this disease (22,27,30), it was necessary to determine whether *B. glumae* was the pathogen causing BPB, as in Japan and other countries, or if it was part of a pathogen complex. Also, the pathogen was reported as seedborne, and rice crops planted with infected seeds can suffer severe losses (31,34). Oxolinic acid is the only chemical that controls this pathogen; however, oxolinic acid-resistant *B. glumae* strains have been isolated from rice in Japan (8), and oxolinic acid is not labeled for use on rice in the United States. In this context, use of pathogen-free seeds is an important practice to reduce or manage the incidence of BPB. Therefore, it was essential to develop rapid, sensitive, and inexpensive methods for identifying and quantifying the levels of *B. glumae* in certified seeds.

Identification of bacterial pathogens based on colony morphology or disease symptoms is difficult and time-consuming (25,28). Final identification must be confirmed with two or more detection methods to ensure that a strain has been identified accurately (11,24). Many biochemical, physiological, and pathological methods have been used to identify and characterize plant-pathogenic bacteria (28). These methods do not identify the pathogens in planta and require pure cultures. However, molecular methods such as real-time polymerase chain reaction (PCR) can be highly sensitive techniques to identify and quantify pathogens both in culture and in planta (13,15,16,27,29). Sequencing of pathogen-specific genes or highly conserved regions such as rRNA or internal transcribed spacer (ITS) regions of RNA is also being used to confirm the identity of pathogen species and to show the diversity within or between species of the same genera (5,10,14,20,23,27).

The objectives of this study were (i) to identify pathogenic bacterial strains from rice with panicle blight, (ii) to determine the role of the different *Burkholderia* spp.

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Fig. 1. Symptoms of bacterial panicle blight disease on rice. **A**, Sheath symptoms: vertical grayish lesion surrounded by dark reddish-brown margin (indicated by arrow). **B**, Panicle symptoms: straw-colored panicles containing florets with a darker base and a reddish-brown line across the floret between darker straw-colored and straw-colored areas. **C**, Typical field symptoms of bacterial panicle blight on a susceptible long-grain rice variety (Trenasse) inoculated with *Burkholderia glumae*. Severely affected panicles remain upright as the grain does not fill.

associated with blighted rice in the southern United States in causing panicle blight, and (iii) to develop techniques for detecting and quantifying BPB pathogen(s) in infected rice seeds.

MATERIALS AND METHODS

Isolation and purification of bacterial strains. Bacterial strains were isolated from diseased sheaths and grains showing symptoms of BPB collected from Louisiana, Mississippi, Arkansas, and Texas rice fields using the semiselective S-PG medium (38). These strains were purified by serial dilution, and single colonies were obtained by replating on S-PG medium. Pure cultures of isolates were stored in King's B broth (KBB) (12) with 30% glycerol at -70°C . Fresh cultures were prepared as needed from frozen stock on plates with King's B agar (KBA). American type culture collection (ATCC) strains of the *Burkholderia* species *B. glumae* (ATCC 33617 and 49703), *B. gladioli* (ATCC 51989 and 19302), *B. plantarii* (ATCC 43733), *B. multivorans* (ATCC BAA-247), and *B. cepacia* (ATCC 10856) were used for comparisons.

Pathogenicity tests. Strains were tested for pathogenicity on rice (*Oryza sativa* L. cv. Cypress) by inoculation at the seedling and heading stages (20 to 30% panicle emergence). As high temperature predisposes rice plants to BPB, pathogenicity tests were carried out in the greenhouse with daytime temperatures ranging between 37 and 41°C and 75 to 95% relative humidity (RH). Inoculum was grown on KBA plates incubated at 30°C for 24 h, then harvested with a sterile cotton swab and suspended in a vial containing 9 ml of sterile distilled water (10^7 to 10^8 CFU/ml). Three to five Cypress seedlings at the three- to four-leaf stage were inoculated by injecting the third leaf sheath with 0.5 ml of the bacterial suspension using 1-ml sterile syringes with BD 23G1 needles. Cypress panicles were sprayed with the bacterial suspension as they were emerging (20 to 30% emerged) from the boot. Sterile distilled water was injected into seedling sheaths or sprayed onto panicles of control plants. Disease reaction was scored on

sheaths and panicles of inoculated plants 2 and 4 weeks after inoculation using the four-category disease scale described in Table 1. After disease scoring, bacterial strains were reisolated from diseased plants, to complete Koch's postulates.

Determining temperature optima. Experiments were carried out separately to determine the optimum growth temperatures for *B. glumae* and *B. gladioli*. Eight PCR-positive virulent strains and two avirulent strains of *B. glumae* isolated from BPB-infected rice panicles from different fields in Louisiana, Texas, and Arkansas, and 10 strains of *B. gladioli* isolated from BPB-infected rice panicles from different fields in Louisiana and Arkansas were used. A 100- μl (OD = 1.0) culture of each bacterial strain was inoculated into 25 ml of KBB and incubated at 30, 35, 40, 45, and 50°C on a rotary shaker at 200 rpm. Three replications were run for each strain. At 24 and 48 h of incubation, 2 ml of bacterial culture was diluted to 8 ml with sterile water, and bacterial growth was measured as absorbance at 600 nm. This experiment was repeated once.

Carbon source utilization and fatty acid analysis of bacterial strains. The ability of strains to use different compounds as sole carbon sources was tested using the Biolog GN2 plates according to manufacturer's instruction (Biolog Inc., Hayward, CA). For initial experiments, the plates were read manually and analyzed using Biolog Software. Later, the selected strains were retested and read using the automated Biolog microplate reader

(Biolog Microstation, Biolog Inc., Hayward, CA) and analyzed with Biolog Microlog1 version 4.01B.

Whole-cell fatty acid analysis of bacterial strains was carried out using the standard extraction procedure for identifying plant-pathogenic bacteria by the MIDI Sherlock Microbial Identification System (25). Extractions and analysis were performed at the Bacterial Identification and Fatty Acid Analysis Laboratory in the Plant Pathology Department at the University of Florida, Gainesville. The fatty acid profiles of our strains were compared with the TSBA50 database. However, there was no entry in the database for *B. glumae*. To identify our strains, 10 pathogenic *B. glumae* strains, collected from Louisiana, Texas, and Arkansas, that had previously been demonstrated to show positive identifications in PCR, ELISA, and Biolog tests for *B. glumae*, were selected, and their fatty acid profiles were used to construct a library entry for *B. glumae* using the Sherlock Library Generation System 4.5. The fatty acid profiles of all subsequent bacterial strains from rice were compared with this *B. glumae* profile.

PCR analyses for ITS region and 16S rDNA. PCR analyses for the 16-23S rDNA ITS regions of *B. glumae*, *B. gladioli*, and *B. plantarii* and 16S rDNA of *B. multivorans* were carried out using species-specific primers (Table 2). DNA was isolated using Bio-Rad InstaGene Matrix kit for PCR ready DNA isolation (Bio-Rad, Richmond, CA), following the manufacturer's instructions. The respective ATCC

Table 2. Primers used in this study for amplifying internal transcribed spacer (ITS) and 16S rDNA regions of suspected *Burkholderia* pathogens

Species name	Forward/reverse primers	PCR product size/refs
<i>B. glumae</i> (ITS)	BGF 5' ACACGGAACACCTGGGTA 3' BGR 5' TCGCTCTCCCGAAGAGAT 3'	400 bp (31)
<i>B. gladioli</i> (ITS)	BLF 5' CGAGCTAATACCGCGAAA 3' BLR 5' AGACTCGAGTCAACTGA 3'	300 bp (5)
<i>B. plantarii</i> (ITS)	BPF 5' AGCCAGTCAGAGGATAAGTC 3' BPR5' CAATTGAGCCGAACATTTAAG 3'	200 bp (31)
<i>B. multivorans</i> 16S rDNA	BMF5' TGTCCGGAAAGAAATCCTTG 3' BMR5' AGCACTCCCGAATCTCTTCA 3'	588 bp (this study)
<i>B. glumae</i> 16S rDNA	16SF 5' AGAGTTTGATCCTGGCTCAG 3' 16SR 5' GGCTACCTGTTACGACTT 3'	1,494 bp (this study)

Table 1. Scoring system for seedling and panicle blight disease based on symptoms caused by *Burkholderia* species in greenhouse inoculation tests and numbers of isolated strains falling into each category

Scale	Seedling		Panicle	
	Symptoms	No. of strains ^a	Symptoms	No. of strains ^a
0	No symptoms produced after inoculation	96	No signs of disease on the panicle or florets	72
+	Slight browning around the injection site	73	Some browning of the florets with most grain filling normally	93
++	Distinct grayish lesion 1-2 cm in diameter or spreading up and down from the injection site with darker brown margin	105	Browning on most florets and some grain not filling, lesions formed on upper most sheath covering panicles	34
+++	Lesion spreading up on the stem from the injection site with the inner leaf blade yellowing or blighted and turning necrotic	90	Panicles poorly emerging, florets discolored or with dark brown base with many florets sterile and panicle tending to remain upright due to lack of grain filling	165

^a Number of strains observed with each disease reaction from 364 strains.

bacterial cultures of each species served as positive controls. PCR reactions were carried out using Promega master mix (Promega, Madison, WI) in a 25- μ l reaction mixture containing 1 μ l of total DNA (150 to 200 ng), 12.5 μ l of 2 $\times$ master mix (DNA polymerase, 0.05 unit/ μ l, 3 mM MgCl₂, 400 μ M each dNTP), 1 μ l of each primer (5 pmole of each forward and reverse), and 9.5 μ l of sterile distilled water. DNA sequences corresponding to the ITS and 16S rDNA regions from the genomic DNA were amplified in a Techne-Genius PCR machine with varying temperature conditions as reported earlier (5,13,30).

PCR reaction of field samples and commercial seed samples. A total of 10 infected florets each from field-collected, naturally infected immature panicles showing BPB symptoms or 20 seeds from commercial seed lots (obtained from the Louisiana State Seed Laboratory) were cut or broken into approximately 0.5-cm pieces, mixed with 3 ml of sterile water, and shaken on a rotary shaker for 2 h at 200 rpm, and then vortexed for 2 min. The bacterial cells were pelleted by centrifugation at $4,350 \times g$ for 10 min. Bacterial DNA was isolated from the pellets as described previously and used in PCR reactions. Seeds were not ground in a mortar to avoid gelling of the pellets by starch from the endosperm.

Cloning and sequencing of 16S rDNA genes and ITS regions. Sequences of both the 16S rDNA gene and 16S-23S rDNA ITS region were determined for representative *B. glumae* and *B. gladioli* strains collected from Louisiana, Texas, and Arkansas, along with their respective ATCC strain controls. Amplification of the 16S rDNA gene was done using 16S rDNA primers (Table 2), and the amplicon was cloned into a pDrive cloning vector (Qiagen, Valencia, CA) according to the manufacturer's instructions. The gene was sequenced using specific primers with the Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and an automated sequencer. For the ITS region, PCR products were gel eluted using Sigma GenEluot agarose spin columns (Sigma, St. Louis, MO), concentrated, and used for sequencing. 16S rDNA and ITS sequences of pathogen and ATCC strains were compared with the GenBank database (BlastN 2.2.20). Sequences were aligned using the Multi-align Program to determine similarity percentages.

DNA isolation for real-time PCR. The ATCC 33617 *B. glumae* strain, our *B. glumae* strains, and other bacterial cultures isolated in this study (Table 3) were transferred to KBB and incubated on an orbiting shaker for 18 h at 200 rpm at 35°C. Bacterial cells (OD₆₀₀ = 0.5) were collected from 1 ml of culture by centrifuging at $8,600 \times g$ for 3 min, and pellets were used for DNA isolation. Total genomic DNA was isolated using the Bio-Rad Aq-

uaPure Genomic DNA kit (Bio-Rad) as per the manufacturer's instruction. Simultaneously, the number of CFU in 1 ml of bacterial culture was enumerated by serial dilution and plating on KBA. Three replications were run for both DNA isolation and population count. The quality of DNA was checked on agarose gel and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE).

Primer, probe design, and optimization of real-time PCR conditions. Real-time PCR primers and Taqman probe (Applied Biosystems) were designed using Primer3 software based on previously published information (31) (GenBank accession no. D87080) and our *B. glumae* strains ITS region sequences (accession numbers DQ097385 to DQ097388). The following forward primer: 5' CAAGATGATTCGAACGCAAGTT 3'; reverse primer: 5' TCGCTCTCCCGAAGAGATGA 3'; and probe: 5' TACGGCACA AATGCGAGAACTCAACCT 3' were used for real-time reactions. The Taqman probe was labeled with 6-carboxy-fluorescein (FAM) at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end. Different probe concentrations (50 to 300 nM), primer concentrations (2.5, 5, 10, 15, and 20 pmole), and annealing and extension times (20, 30, 40, and 60 s) were tested in real-time PCR assays using a uniform template concentration of ATCC *B. glumae* DNA. The quality of DNA and real-time primer pairs was checked using regular PCR. Real-time PCR was performed using Taqman universal PCR master mix with no AmpErase UNG in an ABI prism 7700 sequence detection system (Applied Biosystems). The 30- μ l reaction mixture consisted of 15 μ l of 2 $\times$ master mix, 1 μ l each of forward and reverse primers (5 pmol/ μ l), 1 μ l of probe (100 nM), and varying amounts of DNA template and sterile water. PCR conditions consisted of a 10-min preincubation period with a 15-s denaturation at 95°C leading to 40 cycles of annealing and extension at 60°C for 20 s. In all experiments, the non-template DNA control was maintained as a negative control and three replicates were run for each sample.

Standard curve preparation. DNA isolated from the *B. glumae* ATCC 33671 strain was used for preparing a standard curve. Based on DNA yield from a known quantity of bacterial cells, DNA concentrations of 0.25, 2.5, 25, and 250 pg and 2.5, 25, 250, and 2,500 ng, which corresponded to 10^1 to 10^8 CFU/ml, were used for standard curve preparation. Three replications were used for each concentration. The standard curve was obtained by plotting Ct (threshold cycle) values of each DNA concentration against the log values of DNA concentration which corresponded to the population (cell number). This was subsequently used for quantifying target bacterial DNA from pure cultures and seed

samples with unknown concentrations of pathogen cells.

Real-time PCR identification of bacterial strains and quantification of bacteria from seed samples. DNA isolated from 1 ml of *B. glumae* (ATCC 33617) solution (OD₆₀₀ = 0.5), 7 strains of *B. glumae* from this study, *B. gladioli* (ATCC 19302), *Pseudomonas putida*, *P. aeruginosa*, and *Agrobacterium* sp. were used in real-time PCR to determine the specificity of the probe and primers among species of the same genus or from other genera. Naturally infected panicle samples from grower rice fields and samples of certified rice seed from the Louisiana State Seed Laboratory were used in tests to quantify pathogenic bacteria. Infected seed samples (1, 2, 5, or 10 seeds/sample) were broken into 0.5-cm pieces, transferred into small vials containing 3 ml of sterile distilled water, vortexed for 2 min, and incubated at 39°C at 200 rpm. After 2 h, the vials containing seed suspensions were vortexed for 2 min and 1 ml of seed suspension was withdrawn to isolate the genomic DNA using a Bio-Rad AquaPure Genomic DNA kit. After checking the quality and quantity of DNA, an equal quantity of DNA from each sample was used for real-time PCR. Three replicates were used for each seed sample. Ct values were compared with the standard curve to estimate bacterial populations in seed samples.

RESULTS

Isolation and identification of *Burkholderia* species. A total of 420 bacterial strains were isolated from BPB-infected rice sheath and panicle samples collected from the southern United States during the 1995 to 2004 seasons, including 315, 72, and 33 strains from Louisiana, Arkansas, and Texas, respectively. One *B. glumae* strain was isolated in 2006 from a disease sample obtained from Mississippi. The colony characters of these strains were compared with ATCC strains of several *Burkholderia* species. Most rice strains were similar in colony characteristics on S-PG medium, having circular, smooth, opalescent, convex type colonies with a purple center as previously described (38). The *Burkholderia* colonies were nonfluorescent under UV light and produced a yellow pigment on KBA.

Pathogenicity tests. Pathogenicity tests revealed that 292 strains (80%) of the 364 strains tested were pathogenic on either rice panicles or seedlings. Eighty-eight percent (258 of 292) of the strains caused symptoms typical of panicle blight (Table 1). Seventy-two (25%) of the pathogenic strains were highly virulent, with typical symptoms on both seedlings and panicles (Table 1), while 93 (31%) of the strains caused severe symptoms only on inoculated panicles. The remaining strains produced weak to moderate symptoms either on seedlings or panicles. Most of the

pathogenic strains (>234) were in the genus *Burkholderia*. Symptoms were only produced by *Burkholderia* strains identified as *B. glumae* and *B. gladioli*. The symptoms induced by *B. glumae* and *B. gladioli* were similar, and it was not possible to distinguish between species based on symptoms. Koch's postulates were confirmed by reisolating both *B. glumae* and *B. gladioli* from a few representative disease samples showing typical panicle blight symptoms. Twenty *B. glumae* strains (5%) were not pathogenic. Pathogenic *Burkholderia* strains produced a yellow pigment, identified as toxoflavin, while nonpathogenic strains did not.

Temperature optima. A similar pattern of growth was observed over different

temperatures among virulent and avirulent strains of *B. glumae*, with different growth rates expressed at 30 and 35°C, and similar rates at 40 to 50°C. Growth patterns and rates were very similar among the strains of *B. gladioli* isolated from diseased rice from different locations in the southern United States. The bacterial populations increased as incubation time increased from 24 to 48 h. None of the *B. gladioli* strains grew at temperatures above 45°C. Based on these results, the temperature optima ranged between 38 and 40°C for *B. glumae* and 35 and 37°C for *B. gladioli*. These results were confirmed with a second experiment.

Biolog GN2 identification. Pure cultures of tested strains (364) were first sub-

jected to gram staining and then identified based on carbon and nitrogen substrate utilization in the Biolog GN2 system using manual reading of the Biolog plates. Of these strains, 288 were initially identified as *Burkholderia* species including *B. glumae*, *B. gladioli*, *B. multivorans*, *B. plantarii*, *B. cocovenenans*, and *B. vietnamiensis*. The remaining 76 strains were identified as *Pseudomonas* species, including known pathogens and nonpathogens, or as belonging to 13 other genera (Table 3).

Biolog analyses were then rerun on 15 strains each of the strains identified by manual plate readings as *B. gladioli*, *B. multivorans*, and *B. plantarii*; three strains of *B. vietnamiensis*; two strains of *B. cocovenenans*; and some pathogenic non-

Table 3. Identification of selected bacterial strains isolated from the bacterial panicle blight complex on rice from the southern United States using different methods

Strain no. ^a	Biolog ID	Origin of isolate ^b	Pathogenicity ^c test		Fatty acid identification ^d		
			Seeding	Panicle	MIDI/TSBA (similarity index)	<i>Burkholderia glumae</i> (similarity index)	PCR ^e
80-gr-1	<i>B. glumae</i>	LA	+++	+++	<i>B. cepacia</i> (0.116)	<i>B. glumae</i> (0.883)	<i>B. glumae</i>
189gr-4	<i>B. glumae</i>	TX	+++	+++	No match (0.000)	<i>B. glumae</i> (0.799)	<i>B. glumae</i>
99gr-4-a	<i>B. glumae</i>	LA	++	+++	No match (0.000)	<i>B. glumae</i> (0.936)	<i>B. glumae</i>
99gr-8-a	<i>B. glumae</i>	LA	++	+++	<i>B. cepacia</i> (0.599)	<i>B. glumae</i> (0.945)	<i>B. glumae</i>
99gr-7-b	<i>B. glumae</i>	LA	++	+++	<i>B. cepacia</i> (0.386)	<i>B. glumae</i> (0.910)	<i>B. glumae</i>
99sh-14	<i>B. glumae</i>	LA	+++	+++	<i>B. cepacia</i> (0.564)	<i>B. glumae</i> (0.937)	<i>B. glumae</i>
188gr-2	<i>B. glumae</i>	AR	++	+++	<i>B. cepacia</i> (0.116)	<i>B. glumae</i> (0.774)	<i>B. glumae</i>
190gr-1	<i>B. glumae</i>	TX	+	+++	<i>B. cepacia</i> (0.324)	<i>B. glumae</i> (0.948)	<i>B. glumae</i>
324gr-2	<i>B. gladioli</i>	AR	++	+++	No match (0.000)	<i>B. glumae</i> (0.578)	<i>B. glumae</i>
318gr-4	<i>B. glumae</i>	AR	++	+++	<i>B. cepacia</i> (0.684)	<i>B. glumae</i> (0.851)	<i>B. glumae</i>
99sh-15a	<i>B. glumae</i>	LA	+++	+++	<i>B. cepacia</i> (0.614)	<i>B. glumae</i> (0.944)	<i>B. glumae</i>
99sh-17	<i>B. glumae</i>	LA	++	+++	<i>B. cepacia</i> (0.568)	<i>B. glumae</i> (0.930)	<i>B. glumae</i>
366gr-6	<i>B. glumae</i>	AR	++	++	<i>B. cepacia</i> (0.591)	<i>B. glumae</i> (0.915)	<i>B. glumae</i>
117g1-7a	<i>B. glumae</i>	LA	0	0	<i>B. cepacia</i> (0.578)	<i>B. glumae</i> (0.940)	<i>B. glumae</i>
189gr-8	<i>B. glumae</i>	TX	0	0	<i>B. cepacia</i> (0.245)	<i>B. glumae</i> (0.663)	<i>B. glumae</i>
98gr-1	<i>B. glumae</i>	LA	0	0	<i>B. cepacia</i> (0.769)	<i>B. glumae</i> (0.772)	<i>B. glumae</i>
191sh-10	<i>B. glumae</i>	TX	0	+++	<i>B. cepacia</i> (0.394)	<i>B. glumae</i> (0.828)	<i>B. glumae</i>
117g1-11	<i>B. glumae</i>	LA	0	+++	<i>B. cepacia</i> (0.224)	<i>B. glumae</i> (0.934)	<i>B. glumae</i>
170sh-1	<i>B. gladioli</i>	LA	+	+	<i>B. gladioli</i> (0.450)	No match (0.000)	<i>B. gladioli</i>
217sh-1	<i>B. gladioli</i>	LA	+	++	<i>B. gladioli</i> (0.474)	No match (0.000)	<i>B. gladioli</i>
223gr-1	<i>B. gladioli</i>	LA	++	++	<i>B. gladioli</i> (0.310)	No match (0.000)	<i>B. gladioli</i>
223gr-2	<i>B. gladioli</i>	LA	++	+++	<i>B. gladioli</i> (0.470)	No match (0.000)	<i>B. gladioli</i>
238gr-3	<i>B. gladioli</i>	LA	+++	++	<i>B. gladioli</i> (0.608)	No match (0.000)	<i>B. gladioli</i>
321gr-6	<i>B. gladioli</i>	AR	++	+	<i>B. gladioli</i> (0.293)	No match (0.000)	<i>B. gladioli</i>
372gr-1	<i>B. gladioli</i>	AR	++	+++	<i>B. gladioli</i> (0.799)	No match (0.000)	<i>B. gladioli</i>
378g-3	<i>B. gladioli</i>	AR	++	+	<i>B. gladioli</i> (0.746)	No match (0.000)	<i>B. gladioli</i>
382gr-1	<i>B. gladioli</i>	AR	+++	++	<i>B. gladioli</i> (0.455)	No match (0.000)	<i>B. gladioli</i>
385gr-1	<i>B. gladioli</i>	AR	++	+	<i>B. gladioli</i> (0.816)	No match (0.000)	<i>B. gladioli</i>
156sh-1	<i>Pseudomonas resinovorans</i>	LA	0	0	<i>P. stutzeri</i> (0.518)	No match (0.000)	?
25-2	<i>P. aeruginosa</i>	LA	0	0	<i>P. putida</i> (0.122)	No match (0.000)	?
170gr-4	<i>Agrobacterium</i> sp.	LA	0	0	<i>A. radiobacter</i> (0.848)	No match (0.000)	?
118sh-5	<i>Pseudomonas</i> grp2	LA	0	0	NT	NT	?
189sh-6	<i>Acidovorax avenae</i> subsp. <i>avenae</i>	TX	0	0	<i>A. avenae-avenae</i> (0.939)	No match (0.000)	?
ATCC 33617	<i>B. glumae</i>	Rice/Japan	NT	NT	<i>B. cepacia</i> (0.687)	<i>B. glumae</i> (0.715)	<i>B. glumae</i>
ATCC 49703	<i>B. glumae</i>	Rice/Japan	NT	NT	<i>B. cepacia</i> (0.418)	<i>B. glumae</i> (0.880)	<i>B. glumae</i>
ATCC 51989	<i>B. gladioli</i>	Human USA	NT	NT	<i>B. gladioli</i> (0.652)	No match (0.000)	<i>B. gladioli</i>
ATCC 19302	<i>B. gladioli</i>	Onion USA	NT	NT	NT	NT	<i>B. gladioli</i>
ATCC 43733	<i>B. plantarii</i>	Rice/Japan	NT	NT	<i>B. gladioli</i> (0.412)	<i>B. glumae</i> (0.060)	<i>B. plantarii</i>
ATCC 10856	<i>B. cepacia</i>	—	NT	NT	<i>B. gladioli</i> (0.550)	<i>B. glumae</i> (0.158)	NT
ATCC BAA-247	<i>B. multivorans</i>	Human Belgium	NT	NT	NT	NT	<i>B. multivorans</i>

^a Bacterial strains were isolated either from sheath (sh) or grains (gr). ATCC = American Type Culture Collection (Manassas, VA, USA).

^b Origin of isolate: LA = Louisiana, TX = Texas, AR = Arkansas, or country/host.

^c +, ++, +++ refer to weakly, moderately and highly virulent reactions. 0 = nonpathogenic; NT = not tested for respective analysis.

^d Fatty acid data were first compared with the MIDI data base and later with a newly developed *B. glumae* library. Similarity index in parentheses must be at least 0.50 to be considered as acceptable species identification.

^e All the strains were first screened with *B. glumae* primers, and then all negative strains were again retested with *B. glumae* primers, followed by other primers if not identified (*B. gladioli*, *B. plantarii*, and *B. multivorans*). If negative = ?.

Burkholderia spp., including *Pseudomonas pyrocinia* and *P. tolaasii* using an automated Biolog reader. All of the above strains were then identified as *B. glumae* by the Biolog database.

Fatty acid analysis. Initial fatty acid analyses, using the TSBA50 database of the MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, DE), identified most of our *B. glumae* strains as *B. cepacia* GC subgroup B (Table 3), as the MIDI system did not have an entry for *B. glumae* in its database. Fatty acid profiles of 24 strains matched with the MIDI *B. gladioli* profile. Twelve of these strains did not react with *B. gladioli* species-specific primers in PCR, but did react with *B. glumae* primers. Later, fatty acid data of all strains were rerun against the new *B. glumae* library developed in the Bacterial Identification and Fatty Acid Analysis Laboratory, and we found that most of our strains were *B. glumae* with a high similarity index. The rice *B. glumae* library showed no match for PCR positive *B. gladioli* strains or for the other identified bacterial strains including *P. aeruginosa*, *Agrobacterium radiobacter*, *P. stutzeri*, and *Acidovorax avenae* subsp. *avenae* (Table 3).

A dendrogram was generated using fatty acid profiles of 124 strains including all the ATCC *Burkholderia* species control strains, *Burkholderia* species isolated from rice in this study, *Pseudomonas* spp., and other related genera including *Acidovorax* and *Agrobacterium* isolated from rice. Most of the *Pseudomonas* and non-*Burkholderia* species were clustered (groups A, B, and D), all but three of the *B. gladioli* strains were grouped (group C), and all of the *B. glumae* strains were closely grouped (group E). As this dendrogram was too large to present, 41 representative strains were selected from the original 124 strains tested and used to generate a new dendrogram. These strains accurately reflected positioning of strains in the original. In this dendrogram, all *B. gladioli* and *B. glumae* strains were clustered in separate groups with smaller subgroups showing higher similarities with lesser Euclidean distance and with only distant relationship to related genera, such as *Pseudomonas*, *Acidovorax*, and *Agrobacterium* (Fig. 2).

PCR analyses for ITS region and 16S rDNA. A total of 292 strains, including several *Burkholderia* species and related species from rice in the southern United States, were screened with species-specific primers for *B. glumae*, *B. gladioli*, *B. multivorans*, and *B. plantarii* with their respective ATCC strains as controls. Amplicons of expected size were generated from 242 strains with *B. glumae* primers and 12 strains with *B. gladioli* primers. Except for the type strains of *B. plantarii* (ATCC 43733) and *B. multivorans* (ATCC BAA-247), none of the strains identified as *B. plantarii* or *B. multivorans* by our initial

Biolog readings reacted with *B. plantarii* or *B. multivorans* primers. Thirty-eight strains did not react with any of the four species-specific primers.

Direct isolation of DNA from a test group of field-collected diseased rice panicles and DNA isolated from commercial seed samples reacted only with *B. glumae* primers. Positive PCR identification from direct isolation of DNA from single infected seeds was also demonstrated (data not shown).

16S rDNA and ITS sequence analysis. Sequence analysis of the 16S rDNA gene from four *B. glumae* strains (336gr-1, 189gr-8, 398gr-3, and 99gr-4b), two *B. gladioli* strains (223gr-1 and 321gr-6), and PCR product sequencing of ITS regions of four strains each of *B. glumae* (336gr-1, 226gr-1, 99gr-11, and 189gr-4) and *B. gladioli* (223gr-1, S022-2a, 238gr-3, and 321gr-6), along with the respective type strains, followed by comparison of their nucleotide sequences with the GenBank database, showed more than 99% sequence similarity with already published *B. glu-*

mae and *B. gladioli* sequences. 16S rDNA and ITS sequences for strains of these two bacterial species have been submitted to GenBank (accession numbers DQ355164–DQ355169 and DQ097385–DQ097392, respectively). The 16S rDNA sequence alignment results from the rice *B. glumae* and *B. gladioli* strains, using the Multi-align program, showed high similarity within each species.

Real-time PCR primer and probe specificity. The quality of designed primers was tested with PCR using different concentrations of primers with DNA from *B. glumae* (ATCC 33617) (data not shown). As the concentration of primers increased, formation of primer dimers also increased. The lowest concentration (5 pmole) of primers with no primer dimers was selected for real-time PCR assays. There were no differences observed in Ct values when using different concentrations (50 to 300 nmol) of probe and of annealing and extension times (20, 40, and 60 s). The lowest annealing time of 20 s and 100 nmol (final concentration) probe concen-

Fig. 2. Dendrogram based on whole-cell fatty acid profiles of selected strains of bacteria isolated from rice showing symptoms of bacterial panicle blight along with 5 ATCC strains of *Burkholderia* species (MIDI Sherlock microbial identification system database TSBA50 as well as our newly developed *B. glumae* entry library).

tration was selected as optimum for further assays. The specificity of primers and probe were further tested in real-time PCR, using optimized PCR conditions. DNA isolated from the reference *B. glumae* (ATCC 33617) strain and seven rice *B. glumae* strains (two each from Texas and Arkansas, and three from Louisiana), *B. gladioli* (ATCC 19302), and other related genera *Pseudomonas*, *Acidovorax*, and *Agrobacterium* were tested. Cycle by cycle amplification data showed that the rice strains had the same amplification profile as the *B. glumae* ATCC 33617 control strain (Fig. 3). No fluorescence or little background signal was noticed until the last cycle for the ATCC *B. gladioli* and other species, including the nontemplate control.

Standard curve preparation. Real-time PCR assay with a series of known quantities of the reference *B. glumae* (ATCC 33617) DNA provided a standard curve with a linear relationship between the Ct values obtained and the log values of known quantities of DNA. A correlation coefficient (r^2) value of 0.98 was obtained (Fig. 4). Detection limits were as low as 10^1 CFU/ml. By substituting the Ct values of DNA isolated from *B. glumae*-infected seed samples, the population level of *B. glumae* could be determined.

Quantification of bacterial populations from seed samples. The bacterial population level was quantified from seed extracts from healthy seeds, panicle blight-infected seed lots, and field-infected grain/seed samples. Different seed samples varied in their population levels (Table 4). Of the two known healthy seed samples (lot 1 and lot 2) used for detection, one showed no signal for the presence of bacteria while the other was positive with 260 CFU. Three of four samples of certified seed from different rice fields,

obtained from the Louisiana State Seed Laboratory, were positive for *B. glumae* (Table 4). Seed from panicles freshly collected in the field tended to show high levels of *B. glumae*. Samples with varying numbers of seeds (1, 2, 5, and 10 seeds) showed that the pathogen could be detected and quantified from as little as one seed.

DISCUSSION

This research demonstrated that two pathogenic bacterial species, *B. glumae* and *B. gladioli*, were the major pathogens routinely isolated from panicle blighted rice collected in the southern rice area of

the United States over several seasons. Although rice grain rot, caused by *B. glumae* (formerly *Pseudomonas glumae*), was initially considered a serious pathogen only in Japan, it is now reported in several major rice growing countries of the world including Korea (10), Taiwan (2), Latin America (21,40), Vietnam (33), the Philippines (3), and the Gulf of Mexico rice production area in the United States (20,22,27,30). The pathogen was reported in 2007 from symptomless seeds in China, where the isolated strain caused disease in inoculated rice plants (17). In addition, *B. glumae* was also reported as the causal agent of bacterial wilt in tomato, eggplant,

Fig. 4. Standard curve and its correlation coefficient (r^2) were derived by plotting log values of DNA (10-fold dilutions) from 10^1 to 10^8 CFU/ml of *Burkholderia glumae* (ATCC 33617) and threshold cycle (Ct) values obtained in real-time polymerase chain reaction (PCR) for corresponding concentrations. Ct value refers to the PCR cycle number at which ΔR_n value crossed the baseline.

Fig. 3. Real-time polymerase chain reaction (PCR) assay with *Burkholderia glumae* primers and probe. Relative fluorescence (ΔR_n) of probe obtained in real-time PCR cycle by cycle amplification of seven *B. glumae* strains, *B. glumae* (ATCC 33617), *B. gladioli* (ATCC 19302), and 3 non-*Burkholderia* genera (*Pseudomonas*, *Acidovorax*, and *Agrobacterium*). No signal was detected for *Agrobacterium* sp., *B. gladioli* (ATCC 19302), and nontemplate control (NTC) after 40 PCR cycles.

hot pepper, potato, sesame, and sunflower in Korea (10).

After a severe epidemic in the southern U.S. rice production area in 1995, the pathogen was identified by our laboratory as *B. glumae* in 1996 (30). We isolated more than 400 strains of bacteria from infected rice grains in blighted panicles and sheath lesions collected from rice fields in the southern United States during the period 1995 to 2004. Initial characterization of bacterial strains using various phenotypic characters and the Biolog system suggested that *Burkholderia* species, including *B. glumae*, might be involved, as they were isolated from virtually all infected tissues plated on S-PG medium. As bacterial panicle blight had not been reported in the United States, and as some isolated species had not been reported as pathogens on rice, we thought it necessary to thoroughly identify the strains by using a broad array of methods. Use of a semiselective medium, fatty acid analysis, species-specific PCR, and sequencing of the 16S rDNA and ITS regions of 16S-23S rDNA allowed us to identify the most prevalent bacterial pathogen from blighted rice as *B. glumae* and to show that *B. gladioli* was also a cause of the disease, but of less importance (22). In a recent paper, *B. gladioli* was also reported as a cause of seed rotting in Japan (39).

BPB has the potential to reduce the yield of rice by as much as 75% in severely affected rice, as it causes several types of damage, including inhibition of seed germination, seedling blight, sheath rot, floret sterility, and grain abortion (2,10,20,34,36,40). We observed all of these symptoms in our laboratory, greenhouse, and field tests. In our pathogenicity tests, 165 *B. glumae* strains were more virulent on panicles than on seedlings. This suggests that panicles in the boot or emerging panicles are more susceptible than four-leaf-stage seedlings, as was also observed in Japan and Taiwan (2,36). Some of our *B. glumae* strains were avirulent on both seedlings and panicles. Avirulence among strains of bacterial pathogens from rice is common (4). The avirulent strains identified in our study did not produce the yellow-pigmented toxin toxoflavin on KBA. Phytopathogenic species of *Burkholderia* synthesize a variety of toxins such as toxoflavin, fervenulin, and tropolone (26). Toxoflavin has been shown to be necessary for pathogenicity by *Burkholderia*, causing both seedling disease and grain rot on rice (9,10,19).

Modern bacterial taxonomy involves analysis of multiple characters, including physiological processes such as carbon source utilization, fatty acid profiles, DNA genotyping and diagnostics, and protein composition (4,5,11,12,14). In our study, all the strains identified as *B. glumae* by Biolog were placed into *B. cepacia* GC subgroup B by MIDI (Table 3) because the

MIDI system does not include *B. glumae* in its database. In another study, Biolog identified the onion bacterial soft rot pathogen as *P. fragi*, but later it was identified as *B. gladioli* by fatty acid analysis and 16S rDNA sequencing (14), but in this study, reaction of 12/24 *B. gladioli* strains identified by MIDI with *B. glumae* primers, and not with *B. gladioli* primers, questioned the reliability of fatty acid analyses for this group. This kind of variation in fatty acid analysis for bacterial strains associated with rice panicle disease was also experienced by Cottyn et al. (4). After developing a *B. glumae* profile, using U.S. rice strains, and comparing our strains using fatty acid data for dendrogram cluster tree analysis, it was clear that *B. glumae* and *B. gladioli* were both present as pathogens on blighted rice. Furthermore, the fatty acid profiling results were consistent with the species-specific PCR results, which suggests that the amended fatty acid database with the *B. glumae* library can be safely used for identifying *B. glumae*.

PCR has been used to identify pathogens causing new diseases and even to differentiate species within the same genus (5,18,27,31). More recently, real-time PCR assays have been used for rapid, accurate, and sensitive identification and quantification of pathogens from infected plant tissues (15,16,24,29). PCR and real-time PCR have been used as the main methods for identifying several *Burkholderia* species from soil, water, infected plants, and mammalian cells (5,18,21,22,27,32). In our study, we screened all of the rice strains with *B. glumae*-specific primers and then screened *B. glumae*-negative strains with *B. gladioli*-, *B. plantarii*-, and *B. multivorans*-specific primers. Our PCR results combined with results from other identification methods suggested that two pathogen species, *B. glumae* (76%) and *B.*

gladioli (5%), were the major pathogens causing BPB in infected rice from the southern United States and more recently from Panama (21). ITS region and 16S rDNA gene sequence analysis and comparison further confirmed identification of the strains as *B. glumae* or *B. gladioli*. There were other reports that *B. gladioli* could be isolated from rice (4,5,21,39). Our findings confirm the recent report by Ura et al. (39) from Japan that *B. gladioli* is involved in the panicle blight disease complex. Recently, Sayler et al. (27) reported the occurrence of *B. glumae* in the United States. However, their study included a smaller population size of only 29 strains isolated from Arkansas compared to 420 strains from the three major rice growing states of the southern United States. We are presently conducting phylogenetic studies using these strains of *B. glumae* from the United States, ATCC control strains from Japan, and selected strains from Latin America (in cooperation with Fernando Correa from CIAT) to more closely compare relationships and possibly origins of the pathogen.

Infected rice seeds serve as the primary source of inoculum for BPB (34). To prevent the spread of this disease, planting pathogen-free seed is critical, and commercial seed lots should be tested for the presence of these plant-pathogenic bacteria. Damage can be avoided or reduced by not planting seeds with high levels of bacteria. We developed a real-time PCR assay to quantify *B. glumae* from seed samples using Taqman probe and primer designed for the 16S-23S rDNA ITS region of *B. glumae*. The specificity of our designed probes and primers indicates that they could be used for identification purposes. Similar approaches have been used for detecting other pathogenic bacteria (15,16,29). Recently, Sayler et al. (27)

Table 4. Detection and quantification of *Burkholderia glumae* from infected seed lots

No. of seeds tested from each seed lot ^a	Ct values ^b	Calculated no. of cells ^c
Seed lot #1 (cv. Cypress)		
No. of seeds 1	32.3	3.6 × 10 ⁴
2	31.8	5.2 × 10 ⁴
5	26.3	2.9 × 10 ⁶
10	23.6	2.1 × 10 ⁷
Healthy (10 seeds)	No signal**	ND***
Seed lot #2 (cv. Bengal)	39	2.61 × 10 ²
Healthy (10 seeds)		
Seed lot #3	22.4	5.1 × 10 ⁷
(Field infected-10 seeds)		
Seed lot #4	24.8	7.5 × 10 ⁶
(Field infected-10 seeds)		
Lot #5 (10 seeds)	33.3	1.7 × 10 ⁴
Lot #6 (10 seeds)	No signal	ND
Lot #7 (10 seeds)	38	5.4 × 10 ²
Lot #8 (10 seeds)	35.5	3.4 × 10 ³

^a Seed lots 1 and 2 were obtained from the LSU Agricultural Center Rice Research Station, Crowley, LA, and 3 and 4 were from commercial field samples. Remaining samples (5 through 8) were obtained from the Louisiana State Seed Laboratory.

^b Ct = threshold cycle. **: No fluorescence signal was detected even after 40 polymerase chain reaction cycles indicating the negative reaction; ***: not determined.

^c Numbers of cells were calculated using the formula and taking the log₁₀ value for Ct value - y(integer)/slope and finally multiplied by dilution factor.

reported the use of PCR and real-time PCR to identify *B. glumae* from diseased rice tissue using isolates from Arkansas rice and strains provided by our laboratory. Although our PCR assays identified the presence of *B. glumae* in a single seed, real-time PCR quantified the amount of inoculum present in the single seed or even in apparently healthy seeds, indicating that healthy seeds may carry the bacteria without showing BPB symptoms. Recently, *B. glumae* was isolated from symptomless rice seeds in China (17). Quantification of pathogenic bacteria from rice seeds can be used to determine the concentration of inoculum present in a seed lot. It is necessary to demonstrate the minimum number of bacteria necessary to cause severe disease or visible symptoms, but this is complicated by several environmental factors, mainly temperature and physiological condition of the plant. Our assay can also be used to quantify the bacteria present on the leaf sheath or blade, as the early-stage epiphytic bacterial populations were positively correlated with seed rot disease development in the field (34,37). As PCR could also detect dead cells, further confirmation of the bacterial population using other methods is necessary.

In Louisiana, major epidemics of BPB were observed in 1995, 1998, and 2000, years characterized by record high temperatures. Published reports suggested that high temperature favors the development of disease on rice caused by *B. glumae*, especially nights with long duration of high temperature (20,35,37,38). We determined that the temperature optima for growth for *B. glumae* and *B. gladioli* ranged from 35 to 40°C. This is undoubtedly a factor in disease development in the field. Toxoflavin is not produced by *B. glumae* at temperatures below 28°C and is maximally produced at 37°C (19). *B. glumae* and *B. gladioli* strains that did not produce toxoflavin were avirulent (9,19). It's also possible that high temperature predisposes the plant to BPB, as high night-time temperatures lead to higher respiration in plants with increased utilization of photosynthetic products and an exponential decline in enzyme activity resulting from enzyme denaturation as temperatures increase (1).

In conclusion, the identity of the pathogens causing BPB in rice in the United States was determined and confirmed by several methods. Our newly developed *B. glumae* fatty acid library and real-time PCR assay can be used to identify and quantify the bacterial population in both field-infected tissues and commercial seed lots. This will allow growers to avoid planting infected seeds or to treat the seed with disinfectants to reduce pathogen populations, which should greatly reduce losses to Louisiana rice producers and other rice growers in the southern United States. Also, as the pathogens causing BPB

in U.S. rice are now clearly determined, control measures can be developed for this devastating disease.

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