Comparative Analysis of Two Emerging Rice Seed Bacterial Pathogens

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ABSTRACT

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Seed sterility and grain discoloration limit rice production in Colombia and several Central American countries. In samples of discolored rice seed grown in Colombian fields, the species *Burkholderia glumae* and *B. gladioli* were isolated, and field isolates were compared phenotypically. An artificial inoculation assay was used to determine that, although both bacterial species cause symptoms on rice grains, *B. glumae* is a more aggressive pathogen, causing yield reduction and higher levels of grain

sterility. To identify putative virulence genes differing between *B. glumae* and *B. gladioli*, four previously sequenced genomes of Asian and U.S. strains of the two pathogens were compared with each other and with two draft genomes of Colombian *B. glumae* and *B. gladioli* isolates generated for this study. Whereas previously characterized *Burkholderia* virulence factors are highly conserved between the two species, *B. glumae* and *B. gladioli* strains are predicted to encode distinct groups of genes encoding type VI secretion systems, transcriptional regulators, and membrane-sensing proteins. This study shows that both *B. glumae* and *B. gladioli* can threaten grain quality, although only one species affects yield. Furthermore, genotypic differences between the two strains are identified that could contribute to disease phenotypic differences.

The bacterium *Burkholderia glumae* is associated with bacterial panicle blight (BPB), seedling sheath rot, and grain discoloration of rice (6), a disease that is favored by high nighttime temperature and humidity. It has become a major threat to the stability of rice production (4,5,10,31,34). The main symptoms of panicle infection by *B. glumae* include discolored spikelets, stained grains, and abortion of infected grains (6,34,36). The pathogen can also multiply on seed without causing symptoms; it is unknown whether lack of disease is associated with environmental conditions or plant factors (44).

Like *B. glumae*, *B. gladioli* (previously *Pseudomonas gladioli*) is also associated with bacterial panicle blight and sheath and grain browning in rice in several countries (21–24,36). The two species cause similar symptoms, are often isolated from the same diseased plants (36), and can produce a yellow pigment associated with toxoflavin production (11–13,27). Although the impact of *B. gladioli* on yield has not been previously reported, studies indicate that *B. gladioli* is isolated less frequently from infected rice plants than *B. glumae*, and is less aggressive (24). A few phenotypic differences have been identified between plant responses to the species; *B. gladioli* but not *B. glumae* activates host genes encoding chitinases, a process thought to help bacterial competition with plant-associated fungi during infection (17).

The individual and combined contributions of *B. glumae* and *B. gladioli* to BPB symptoms in field conditions are poorly

understood. Impacts on rice yield losses are roughly estimated based on evaluation of panicle sterility or grain weight reduction at harvest time. However, this approach lacks accuracy because multiple factors could affect the plant along the reproductive cycle, including abiotic and biotic stressors. Assessment of yield reduction due to infection by each *Burkholderia* sp. under controlled conditions is needed to understand the potential of each bacterial species to reduce rice yield but has not been performed.

Although the two bacterial species share tissue preference and appearance of disease symptoms, relatively little is known about the genes that contribute to disease development. The phytotoxin toxoflavin, the LipA lipase, and type II and type III secretion systems affect virulence in *B. glumae* (15,32). Although many *B. gladioli* strains also produce toxoflavin, the function of these virulence factors in *B. gladioli* has not been studied. Recently, published genome sequences (8,18,30) could provide key clues for understanding the *Burkholderia* pathogenicity process and for guiding breeding strategies for the development of rice varieties with better tolerance to the disease. Both *B. glumae* and *B. gladioli* produce symptoms in other plants such as onion and gladiolus (36), and both species cause lung infection in immune-compromised humans (2,39). Yet, it is still not understood what genetic factors, if any, separate human- and plant-pathogenic strains. Complete genome sequences of *B. glumae* strain BGR1 and *B. gladioli* strain BSR3 are published (18,30), and draft sequences of *B. glumae* strains LMG2196 and AU6208 were recently added to GenBank. Therefore, basic comparative analyses could help identify differences in virulence function between these closely related species.

In this article, we present the first study directly comparing pathogenicity and yield impacts of South American field isolates of *B. glumae* and *B. gladioli*. We demonstrate that, whereas both *B. glumae* and *B. gladioli* can be isolated from diseased or

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symptomless field seed samples, the two species differ greatly in their capacity to reduce rice yield under controlled conditions. We also generated and analyzed draft genome sequences of Colombian isolates of *B. glumae* (3252-8) and *B. gladioli* (3848s-5), comparing them with one another and with the sequence of the *B. glumae* strains BGR1, AU6208, and LMG2196 and the *B. gladioli* strain BSR3, to identify putative genetic factors that might contribute to differential virulence phenotypes between the species.

MATERIALS AND METHODS

Rice seed samples. Rice seed samples were contributed by farmers or extension personnel in 12 states of Colombia in 2007 to 2011. The samples were collected from fields showing panicle sterility symptoms or yield reduction. From 488 samples received, 100 were randomly selected and processed for *B. glumae* and *B. gladioli* detection using polymerase chain reaction (PCR).

Isolation and purification of bacterial strains from seed samples. Ten seeds were selected from each field sample. Each sample was macerated using a mortar sterilized by irradiation under UV light at 80,000 $\mu\text{J}/\text{cm}^2$. Each powdered sample was resuspended in 500 μl of sterile water, homogenized by vortexing for 5 min, and centrifuged for 5 s. The supernatant was quickly transferred into a new 1.5-ml Eppendorf tube and was centrifuged at 10,000 rpm for 5 min to concentrate the bacteria. The pellet was resuspended in 100 μl of sterile water and then serially diluted up to 10^{-2} using sterile water. Each dilution was plated on King's B agar (KBA) (16) and incubated at 28°C for 48 h. After incubation, bacterial colonies were examined under the stereoscope, and colony types similar to *B. glumae* (pigmented and nonpigmented strains) were selected and transferred to a new KBA plate for purification. After 48 h of incubation, single colonies from each plate were placed in 3 ml of L-Broth medium (1.0% Bacto-tryptone, 0.5% Bacto-yeast extract, and 1.0% NaCl, pH 7.0) and incubated on a shaker at 250 rpm at 28°C for 48 h.

All strains selected for further studies are originally from Colombia and are part of the International Center for Tropical Agriculture (CIAT) bacterial collection maintained in 30% glycerol at -80°C . *B. glumae* 3252-8 and *B. gladioli* 3848s-5 were used for draft genome sequencing and assembly. *B. glumae* (3200-12) and four *B. gladioli* strains (3704-18, 4228-2, 4194-4, and 3848s-5), were used for pathogenicity tests on *Oryza sativa* subsp. *indica* 'Colombia XXI' (CIAT-FLAR gene bank GID 2272556).

Confirmation of bacterial species by amplification and sequencing of internal transcribed spacer 16S-23S ribosomal DNA region. PCR analyses for the 16-23S ribosomal DNA (rDNA) internal transcribed spacer (ITS) region sequences of both *B. glumae* and *B. gladioli* were carried out using species-specific primers (9,28).

For each field isolate, a 100- μl bacterial suspension was incubated at 95°C for 8 min and centrifuged at 10,000 rpm for 3 min. The supernatant (3 μl) was then used directly as a DNA template for PCR. All amplification reactions were performed in a 15- μl final volume containing 1 $\times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM MgCl_2 , 200 μM dNTPs (Promega Corp., Madison, WI), 1 U of *Taq* polymerase (Promega Corp.), and 0.1 μM each primer. PCR reactions were performed in an MJ Scientific PTC-100 machine. The amplification profile consisted of an initial denaturation at 94°C for 2 min; followed by 29 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min; with a final extension at 72°C for 10 min. In addition to the rDNA region, we also used PCR primers from the gyrase gene (*gyrB*) sequences for specific detection of *B. gladioli* described by Maeda et al. (20). PCR for *gyrB* was performed with one cycle at 94°C for 2 min and 29 cycles each at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. A 10- μl aliquot of the reaction was

analyzed by electrophoresis on a 1.2% agarose gel (Invitrogen Co., San Diego, CA) at 100 V for 2 h in Tris-borate-EDTA buffer. Gels were stained with SYBR Safe DNA Gel Stain (Life Technologies, Grand Island, NY).

PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega Corp.) following the manufacturer's instructions. The purified PCR products were sequenced using species-specific primers (20). The sequencing reaction was carried out using 2 to 5 μl of amplification product, with 1 μl of primer at a concentration of 4 M, 2 μl of kit mix (ABI prism BigDye terminator Cycle sequencing kit), and adjusting the volume to 7 μl with sterile water. The reaction was run in a thermocycler PTC 100 (MJ Research, Inc.) using the following profile: 96°C for 20 s, 50°C for 10 s, and 60°C for 4 min for 25 cycles. Subsequently, the PCR products were purified with 3 M sodium acetate (pH 5.5) and 100% ethanol. The sequence reaction was loaded into a sequencing gel on ABI PRISM 377 DNA Sequence system.

The identity of each sequence was verified using the GenBank (BLAST-N and BLAST-X) database (BLAST version 2.0 or 2.1) from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Pigment production. Relative pigment production by *B. glumae* (3200-12) and four strains of *B. gladioli* (3704-18, 4228-2, 4194-4, and 3848s-5) were measured. Each strain was streaked for colony isolation on KBA (16) solid medium for 2 days at 28°C. Bacterial cultures were grown from single colonies in 2 ml of KB liquid medium on a rotary shaker at 100 rpm for 2 days at 28°C. Cells were harvested by centrifugation (10,000 rpm, 5 min). The supernatant (300 μl) was measured at 390 nm on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT), using the Gen 5 software to monitor the results (Biotek).

Inoculum preparation and plant growth for pathogenicity tests. Bacterial strains were grown on KBA medium, incubated at 28°C for 48 h, then removed with a loop and suspended in a vial containing sterile distilled water. The bacterial suspension was adjusted to an optical density at 600 nm of 0.2 using a Spectronic 20 spectrophotometer (Thermo Fisher Scientific, Madison, WI), corresponding to roughly 1×10^8 cells/ml.

Plants of susceptible Colombia XXI rice were grown in plastic pots (16 cm in diameter by 14 cm deep, two plants per pot) until flowering. Fully emerged panicles were inoculated with the bacterial suspension. Four panicles were inoculated with each bacterial strain, one on each of the two plants in each of two pots. Each panicle was sprayed with 3 ml of the bacterial suspension using an airbrush (Paasche, Chicago, IL). Sterile water was used as negative control. Inoculated plants were incubated for 24 h inside a sealed plastic chamber (0.6 m by 0.6 m wide by 1.20 m high) to build a high-humidity environment. After incubation, plants were removed from the chamber and placed in the greenhouse in a random distribution. Ambient temperature and relative humidity (RH) in the greenhouse were recorded hourly with data recorders (HOBO H8 Pro Series Part Number H08-032-08; Onset Computer Corp., Bourne, MA), operated by a computer software program (BoxCar Pro 4.0 Starter Kit, Part Number BCP4.0-ON; Onset Computer Corp.). During the survey, the average temperature and RH were 25.5°C (range 22.1 to 28.7°C) and 83.6% (range 72.2 to 95.9%), respectively.

B. glumae and *B. gladioli* disease development were evaluated at 4 weeks after inoculation. Disease damage was expressed as the percentage of grains with visible disease symptoms, calculated from the total grains obtained from the four panicles of each inoculation treatment. Once the percentage of damage was calculated, the same samples were subjected to a second analysis to evaluate the effect of each *Burkholderia* sp. on yield. This parameter was estimated by comparing the weight of 100 infected grains with the weight of 100 grains from the sterile water control.

All data were analyzed by standard analysis of variance, followed by Duncan's multiple range test using Statistical Analysis System Software (SAS version 9.2; SAS Institute, Cary, NC). Differences were considered significant at the $\alpha = 0.05$ probability level.

Draft genome sequencing and assembly. DNA from *B. glumae* strain 3252-8 and *B. gladioli* strain 3848s-5 was extracted using a standard cetyltrimethylammonium bromide extraction method (25). The DNA was sheared, a library was constructed (Illumina, San Diego, CA), and the DNA was sequenced (55-bp paired-end reads) using an Illumina Genome Analyzer IIx (Illumina) at the Michigan State University Research Technology Support Facility. In all, 73.4 M (4.0 Gbp) purity filtered reads were generated for *B. glumae* 3252-8 and 74.9 M (4.1 Gbp) purity filtered reads were generated for *B. gladioli* 3848s-5. A custom PerlScript was used to remove reads with low-complexity and low-quality regions. The low-complexity threshold was defined as >85% of the base composition composed of a single nucleotide or more than one 'N' present in a read. The low-quality threshold was one or more bases with a quality score <20. In cases where one read passed the cleaning process and the other paired read failed, the entire read pair was discarded. The Velvet short read assembler v1.0.18 (43) was used to generate the assembly. The entire set of cleaned paired-end reads for each genome was used as input for Velvet. The Velvet parameters used in the final assembly of both genomes were a hash length of 33, expected coverage and k-mer coverage cutoff set to "auto", and a minimum contig length of 200 bp. The insert lengths used for the assembly of *B. glumae* 3252-8 and *B. gladioli* 3848s-5 were 345 and 372 bp, respectively.

The assembly statistics for the draft genome of *B. glumae* strain 3252-8 and *B. gladioli* strain 3848s-5 are listed in Supplemental Table 1. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AQOM000000000 (*B. glumae* strain 3252-8) and AQOL000000000 (*B. gladioli* strain 3848s-5).

Functional comparison. To estimate the level of conservation between the genomes of Colombian *B. glumae* and *B. gladioli* strains and the genomes of *B. glumae* strain BGR1 and *B. gladioli* strain BSR3, the nucleotide coding sequences predicted in each plasmid and chromosome of BGR1 and BSR3 were aligned to the assemblies of the Colombian strains using BLASTn (1) (cutoff = >85%). GenBank accessions of BSR3 chromosomes and plasmids are NC_015381, NC_015382, NC_015376, NC_015377, NC_015378, and NC_015383; accessions of BGR1 are NC_012724, NC_012721, NC_012723, NC_012720, NC_012725, and NC_012718.

Lists of precomputed differences in the predicted coding sequences of Asian *B. glumae* strain BSR3 and *B. gladioli* strain BGR1 were obtained from the *Burkholderia* Genome Database using the reciprocal best BLAST hit comparative search function (<http://www.burkholderia.com/geneSearch.jsp>) (40) and from the Microbial Genome Database (MBGD), which uses the DomClust program for ortholog classification (<http://mbgd.genome.ad.jp/>) (35). Comparative analyses are based on the MBGD list. BSR3- and BGR1-specific coding sequences falling into 13 clusters of orthologous groups (COGs) associated with major facilitator superfamily proteins, methyl accepting chemotaxis receptors, and

transcriptional regulators were extracted from the BSR3 and BGR1 genomes using a custom PerlScript. Conservation of these genes in draft genome assemblies of *B. glumae* strains 3252-8, AU6208 (accession AMQT000000000), and LMG2196 (accession AMRF000000000) and of *B. gladioli* strain 3848s-5 were assessed based on the presence or absence of a BLASTn hit in the assemblies (cutoffs, $e = 0.0001$ and 80% nucleotide identity). Genes with predicted involvement in the type III secretion pathway or toxin production were identified based on BLAST similarity to genes previously reported in BGR1. Other validated type III effector gene sequences were downloaded from T3DB (http://biocomputer.bio.cuhk.edu.hk/T3DB/function_download.php) (38) for assessment of the presence in *B. glumae* and *B. gladioli* genomes.

Type VI secretion gene sequences were extracted from the four draft *Burkholderia* genomes, BGR1, and BSR3 by BLASTn alignment to the sequence of *evpA/vipA* from *B. thailandensis* (accession BTH_I2964). BSR3 and BGR1 sequences corresponded to annotated genes (protein accessions are YP_004362835, YP_004348376, and YP_004359059 for BSR3 and YP_002907865, YP_002908402, YP_002908734, YP_002907919, and YP_002910292 for BGR1). Predicted protein sequences were aligned by CLUSTALW (3) with the VipA homologs from *B. thailandensis* strain E264, *B. pseudomallei* strain K96243, *Vibrio cholerae* serovar O1/ATCC39315, *Agrobacterium tumefaciens* strain C5A/ATCC33970, and *Ralstonia eutropha* strain JMP134 (29). Phylogenetic analysis was performed by neighbor-joining analysis of 125 amino acid residues after gap elimination, with distances calculated using the Poisson correction method, using the default settings of the MEGA5 program (33). Sequence alignments and phylogenetic trees were deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloWS/study/TB2:S14408>).

RESULTS

Bacterial isolation from field samples and detection by PCR. The symptoms observed on collected seed samples ranged from high levels of grain discoloration and darkening to samples without apparent symptoms (Table 1; Fig. 1). Due to the high prevalence of these symptoms on the tested samples, darkening and discoloration were considered the most accurate visual indicators of bacterial infection. Grain "darkening" is defined as brown-colored filled grains while the term "discoloration" is used to describe sterile seed with discolored palea and lemma due to bacterial infection. PCR-based pathogen detection on 100 selected rice seed samples revealed that 54% overall were positive for *B. glumae* and 14% for *B. gladioli*. *B. glumae* and *B. gladioli* were detected on symptomless seed samples, with 3 of the 14 symptomless samples positive for *B. glumae* (21.4%) and 2 positive for *B. gladioli* (14.3%). Of the rice samples showing symptoms of grain discoloration, 88.6% tested positive for *B. glumae*, whereas the frequency of *B. gladioli* was similar to that of symptomless seed (15.9%). Analysis of grains showing only brown coloring symptoms showed low frequency of each species, 28.6% for *B. glumae* and 11.9% for *B. gladioli* (Table 1). Interestingly, only a few samples showed coexistence of both bacteria independent of the symptom category, 3% for grain discoloration and 1% for

TABLE 1. Frequency of detection of *Burkholderia glumae* and *B. gladioli* associated with different symptoms in 100 naturally infected field samples

Symptom	Number tested ^y	Number (%) of positive seed samples detected by polymerase chain reaction ^z			
		<i>B. glumae</i>	<i>B. gladioli</i>	Both	Neither
Grain discoloration	44	35 (79.5)	4 (9.0)	3 (6.8)	2 (6.8)
Grain darkening	42	11 (26.1)	4 (9.5)	1 (2.3)	26 (61.9)
Symptomless	14	2 (14.3)	1 (7.1)	1 (7.1)	10 (71.4)
Total	100	48 (48)	9 (9)	5 (5)	38 (38)

^y Number of tested samples.

^z As described by Furuya et al. (9) for *B. gladioli* and Sayler et al. (28) for *B. glumae*.

both grain darkening and symptomless seed. The same analysis confirmed a difference in geographical distribution of both species; although *B. gladioli* was found in only 5 states, *B. glumae* was detected in samples from all 12 states analyzed (data not shown). A subset of 35 *B. glumae* strains isolated in different states were characterized at a pathogenic level on Colombia XXI. No major differences were observed on disease severity among the tested strains (data not shown). After this analysis, *B. glumae* 3200-12 and 3252-8 were randomly selected for further analysis.

After 48 h of incubation on KBA medium, *B. glumae* strains ($n = 35$) appeared as convex colonies with a pronounced halo zone, usually with irregular margins, and an average diameter of 0.6 mm. By contrast, *B. gladioli* colonies were slightly domed, smooth, and shiny, with regular, circular margins and a larger average diameter of 1.6 mm. In addition to one *B. glumae* isolate, four *B. gladioli* isolates from different sites in Colombia were selected for pathogenicity studies (3704-18, 4228-2, 4194-4, and 3848s-5), and the identity of these was confirmed by sequencing the ITS region.

PCR products obtained from four *B. gladioli* strains (3704-18, 4228-2, 4194-4, and 3848s-5) were confirmed to have 98 to 99% sequence homology with the corresponding *B. gladioli* sequence in GenBank (with accession number CP002600). ITS region sequences of *B. gladioli* strains 3704-18, 4228-2, 4194-4, and 3848s-5 have been deposited in GenBank under the accession numbers JF323578, JF323579, JF323580, and JF323581, respectively.

Symptom development by *B. glumae* and *B. gladioli* under controlled conditions. Panicles of Colombia XXI rice that were artificially inoculated with *B. glumae* started to show grain darkening and discoloration 3 to 4 days after inoculation. These symptoms progressed to empty discolored grain, grain staining,

sterility, and abortion of the grains. At 14 days after inoculation, the panicles stayed upright, instead of showing the normal drooping, due to low grain weight. Lesions with brown margins were observed on the flag leaves of inoculated panicles; these symptoms were only occasionally observed in the field. At 30 days after inoculation, affected panicles contained a mixture of green, tan, discolored, and brown grains. The color of the spikelets changed, and ranged from grayish-brown to straw colored with dark-brown bases. Florets showed dark gray or brown bases but the rachis of the panicle remained green (Fig. 1A). In general, plants artificially inoculated with *B. glumae* developed symptoms similar to those commonly observed in the field (24). Symptoms caused by *B. gladioli* included grain sterility and discoloration, with a lower proportion of affected seed compared with *B. glumae* infection (Table 2). Grain filling was not severely affected by *B. gladioli* but affected grains appeared mainly as dark-brown seed (Fig. 1B). *B. gladioli* symptoms also took a longer time to develop than those caused by *B. glumae*, appearing 4 to 7 days after inoculation. None of the above symptoms were observed on seed from mock-inoculated panicles up to 30 days after inoculation (Fig. 1C).

We evaluated grain weight on inoculated panicles to quantify yield reduction caused by each species. *B. glumae* reduced grain weight up to 75% relative to control plants (Table 2). On the other hand, *B. gladioli* had little effect on yield, suggesting that the primary importance of this species may be grain quality. Overall, these results showed that *B. glumae* (strain 3200-12) is more aggressive than any of the tested strains of *B. gladioli* ($P < 0.05$). Among strains of *B. gladioli*, the proportion of seed damaged was 55.7 to 79.6%.

Pigment production was determined by observing colony color of all field isolates and by spectrophotometric measurement of

Fig. 1. Symptoms of bacterial panicle blight on rice seed under greenhouse conditions evaluated at 30 days after inoculation. **A**, Seed samples infected by *Burkholderia glumae*; **B**, seed samples infected by *B. gladioli*; and **C**, seed treated with sterile water.

TABLE 2. Characteristics of strains of *Burkholderia glumae* and *B. gladioli* derived from this study^w

Species, strain	Geographical origin	Symptom type seed	Pigment production ^x	Damage (%) ^y	Adjusted weight to 14% MC ^z	Reduction of yield (%)	Sequence
<i>B. glumae</i>							
3200-12	Monteria	Grain discoloration	0.29 c	93.8 a	0.60 b	75.6	No
3252-8	Antioquia	Grain discoloration	ND	ND	ND	ND	Yes
<i>B. gladioli</i>							
3704-18	Palmira	Grain darkening	1.97 a	73.1 bc	1.91 a	22.4	No
4228-2	Venadillo	...	0.53 bc	55.7 c	2.26 a	8.1	No
4194-4	Ibague	...	1.22 ab	79.6 ba	2.44 a	0.8	No
3848s-5	Santa Rosa	...	0.41 bc	78.7 ba	2.31 a	6.1	Yes
Sterile water	...	Symptomless	0.25 c	0.0 d	2.46 a	0	...

^w Different letters in same column indicate significant differences at $P < 0.05$ (analysis of variance). ND = not determined.

^x Mean of two biological replications. Letters correspond to grouping by Tukey-Kramer method ($P < 0.05$).

^y Number of affected grains from the total (average of four panicles per strain for three replications).

^z Weight in grams of 100 seeds at 14% moisture content.

supernatants from the isolates tested for pathogenicity. *B. glumae* colonies generally had similar levels of pigmentation, although a few strains lacked pigmentation entirely. Meanwhile, *B. gladioli* colonies ranged from pale, medium, to dark yellow in color. The level of damage produced by strains in the pathogenicity study did not have a clear relationship to the level of pigment produced in liquid media (Table 2). For example, *B. gladioli* strain 3848s-5 produced a relatively low level of pigment but was comparable with more highly pigmented isolates in terms of numbers of affected seed and overall yield loss, whereas *B. gladioli* strain 3704-18 produced the highest pigment levels and showed reduced capacity to affect yield compared with the less-pigmented *B. glumae* strain 3200-12.

The draft genomes of Colombian isolates of *B. glumae* and *B. gladioli*. Draft genomes of *B. gladioli* strain 3848s-5 and the *B. glumae* strain 3252-8 were sequenced and assembled de novo. Features of the assembly are summarized in Supplemental Table 1 in comparison with four other publicly available completed or draft genomes of *B. glumae* and *B. gladioli*. The draft assemblies reported here are comparable with the publicly available draft genomes of *B. glumae* strains LMG2196 (the type strain, isolated from rice) and AU6208 (a human clinical isolate, also pathogenic on rice) in size, median length of contigs, and GC content. The total sequence length of each draft assembly is shorter than completed genomes of the same species, which could arise from underrepresentation of repetitive sequence in the draft assemblies.

A previous comparative analysis between the genome of *B. glumae* strain BGR1 and the draft assembly of the U.S. *B. glumae* strain 336gr-1 found evidence of extensive genomic plasticity between genomes, with a substantial amount (6.6%) of the BGR1 genome being unique (8). Alignment of predicted BGR1 coding sequences to the draft genome of Colombian strain 3252-8 showed that 94% of BGR1 coding sequences on chromosome 2, the location of the majority of characterized virulence genes, are conserved in the 3252-8 assembly, whereas only 84% of chromosome 1 coding sequences are conserved (Supplemental Table 2). Conservation of BGR1 coding sequences from the four plasmids was lower (40 to 70%). In *B. gladioli*, 90 and 98% of coding sequences in chromosome 1 and 2 of strain BSR3, respectively, are present in the strain 3848s-5 assembly (Supplemental Table 3). Despite increased conservation of chromosomal genes, only 56 to 60% of the genes in *B. gladioli* plasmids 1 and 2 have hits in the 3848s-5 draft genome, whereas few or no genes in plasmids 3 and

4 are found in the assembly. It is not known whether Colombian *B. gladioli* isolates lack plasmid 3 and 4 or whether these plasmids were specifically lost from strain 3848s-5 during lab culture. Although some of the interstrain differences may result from gaps in the draft assemblies, these results support previous findings that the genomes of *B. glumae* include substantial plasticity, with the greatest conservation in chromosome 2 and the lowest conservation among the plasmids. Our results suggest similar levels of sequence diversity among *B. gladioli* strains.

Functional comparison between *B. glumae* and *B. gladioli* genomes. In addition to the Colombian isolate genomes reported here and the completed genomes of BGR1 and BSR3, the draft genomic sequence of two additional strains of *B. glumae* are now available. Identifying genetic differences between *B. glumae* and *B. gladioli* core genomes could provide important clues to the basis of the pathogenicity differences we observed between the two species. To lay a framework for this type of study in the future, we analyzed selected major differences in predicted gene content among the genomes. Lists of coding sequences of *B. glumae* BGR1 with no ortholog in *B. gladioli* BSR3, and vice versa, were obtained from two online resources (the MBGD and the *Burkholderia* Genome Database) that use different ortholog prediction methods. Both analyses arrived at similar numbers and composition of *B. glumae*- and *B. gladioli*-specific predicted coding sequences, with roughly 78% of *B. glumae* genes having orthologs in *B. gladioli* and 63% of *B. gladioli* genes having orthologs in the smaller *B. glumae* genome. The composition of the six most prevalent predicted functional categories of these species-specific genes as calculated by the MBGD is shown in Figure 2. Genes with predicted involvement in uptake and metabolism of small molecules (amino acids and secondary metabolites) form the largest categories of interspecific differences, with major facilitator superproteins and methyl-accepting chemotaxis receptors the two most frequent gene families represented. Predicted transcription factors, including response regulators of two-component signal transduction systems, composed the second-largest group of *B. glumae*- and *B. gladioli*-specific differences. Although these are broad functional categories with many members, small molecule receptors and transcriptional response regulators are known to greatly impact environmental sensing and host adaptation abilities of bacterial plant pathogens (37,42). To determine the level of conservation of these classes of BSR3- and BGR1-specific genes among other isolates, the sequences of the pre-

Fig. 2. Functional categorization of the seven largest categories of predicted genes specific to *Burkholderia glumae* strain BGR1 and *B. gladioli* strain BGR3.

dicted species-specific major facilitator superfamily proteins, methyl-accepting chemotaxis receptors, and eight families of transcriptional regulators were aligned with the draft genomes of *B. glumae* strains 3252-8, LMG2196, and AU6208 and the *B. gladioli* strain 3848s-5. The composition of “core” differences among these classes of genes (i.e., the genes conserved among all *B. glumae* but that are not detected in *B. gladioli* and vice versa) is listed in Table 3. Most of the genes differentiating BSR3 and BGR1 are conserved among the other members of each species; the presence and absence of individual family members in each draft genome are detailed in Supplemental Tables 4 and 5.

Another prevalent category of genes with unique orthologs specific to both *B. glumae* and *B. gladioli* genomes is that of type VI secretion systems (T6SS). T6SS function to secrete proteins into media or host cells and have demonstrated roles in plant pathogenesis of several bacteria (26). Numerous phylogenetic families of T6SS have been described in diverse genomes; of the six families characterized in the human pathogens *B. pseudomallei* and *B. thailandensis*, T6SS-5 is required for full virulence on a mouse host and T6SS-1 is important for persistence in environmental biofilms (29). By aligning sequences of *B. glumae* and *B. gladioli* homologs of VipA, a conserved protein sequence previously used for T6SS phylogenetic analysis (29), we analyzed the composition of T6SS families encoded in the six genome sequences of *B. glumae* and *B. gladioli* strains. Phylogenetic analysis suggests that *B. glumae* has four distinct families of T6SS genes, whereas *B. gladioli* has three. Although both species encode T6SS genes similar to T6SS-1 of *B. pseudomallei*, only *B. glumae* strains encode genes in the clades of T6SS-2 and T6SS-5, as well as another putative family related to a T6SS from *R. eutropha* (Fig. 3). Two additional T6SS clades are found only in *B. gladioli*, including one similar to T6SS-3 and a novel clade equally related to T6SS-2, -3, and -6. Although the T6SS families found in *B. glumae* did not differ among the four species tested, the T6SS-3-like clade was found in *B. gladioli* strain BSR1 but not 3848s-5. These results suggest that, of up to six T6SS families found among *B. glumae* and *B. gladioli*, only one is shared between the species. Due to the fragmented nature of available *B. glumae* and *B. gladioli* draft genomes, further sequencing and PCR validation are needed to confirm whether each T6SS is complete and likely functional in each of these strains.

Finally, we analyzed the sequence of several demonstrated or likely *B. glumae* virulence factors in each of the rice-pathogenic *Burkholderia* genomes. *B. glumae* produces the essential pathogenicity factor toxoflavin through the *toxABCDE* operon, under transcriptional control of the LysR-family transcriptional regulator ToxR (32). The LuxR-family quorum-sensing regulator TofR also has a role in controlling toxoflavin production, as well as expression of another gene involved in virulence, the lipase gene *lipA* (7). Alignment of *tox* operon genes *tofR*, and *lipA* from

BGR1 to the five other *B. glumae* and *B. gladioli* genomes showed these genes to be universally present (Table 4) and highly conserved among both species.

Although the substrates of type III secretion in *B. glumae* are not known, the *hrp* type III secretion system (T3SS) is essential for full virulence and a hypersensitive response on nonhost plants (15). The genomes of *B. glumae* BGR1 and *B. gladioli* BSR3 contain five and four predicted genes, respectively, annotated as putative type III-secreted effector genes. Both genomes are predicted to encode genes for the effector HrpK1, a second putative effector with an Hpr kinase domain, and an Awr-family protein. The presence of the three conserved effector genes was detected in each of the four *B. glumae* and *B. gladioli* draft genomes; the exception is the Awr-family effector gene, which is missing in the assembly of strain AU6208 (Table 4). *B. glumae* BGR1 also encodes predicted homologs of the *Erwinia* effector Eop3 and the harpin/pectate lyase HrpW, both of which are present in all four *B. glumae* genomes but in neither *B. gladioli* genome. *B. gladioli* BSR3 encodes a YgbK domain protein that is a putative type III effector, and this gene is also present in *B. gladioli* 3848s-5 but in none of the *B. glumae* genomes (Table 4). To determine whether additional type III effector genes may be present in rice-pathogenic *Burkholderia*, we searched for alignment matches to the T3DB database of 330 experimentally validated effector and translocon genes from 26 genera of bacteria but no effector candidates were found. Although there may be additional type III-secreted effectors undiscovered in rice-pathogenic *Burkholderia* spp., these results show that the pool of secreted effectors is likely very small or distinct from known effectors, and that the composition of predicted effectors differs between the species.

Fig. 3. Neighbor-joining phylogenetic analysis of predicted VipA protein sequences in *Burkholderia glumae* and *B. gladioli* strains. VipA families from *B. glumae* strains are boxed in solid lines and those from *B. gladioli* strains are boxed in dashed lines. Classes of type VI secretion systems (T6SS) previously named in *B. pseudomallei* and *B. thailandensis* are indicated to the right. Bootstrap test percentages (1,000 replicates) are shown next to the branches. Units represent amino acid substitutions per site.

TABLE 3. Common gene families with members specific to four *Burkholderia glumae* or two *B. gladioli* genomes

Functional category, gene family	Number of genes specific to genomes of	
	<i>B. glumae</i>	<i>B. gladioli</i>
Membrane transporters		
Major facilitator superfamily	7	64
Signaling receptors		
Methyl-accepting chemotaxis	10	4
Transcriptional regulators		
LysR	6	102
AraC	7	55
MarR	2	6
LuxR	1	1
AsnC	2	0
MerR	1	1
Cnp	1	3
GNTR	1	5

DISCUSSION

Grain sterility is becoming an important limitation for rice production in United States and Latin America (21,28,31). We detected the presence of *B. glumae* and *B. gladioli* in symptomatic and asymptomatic field samples collected in different locations of Colombia. To compare the relative roles of these two pathogens on rice yield and quality, we developed a controlled greenhouse assay to assess the individual capacity of each bacterial pathogen to inhibit grain filling process. Evaluation and identification of the symptoms produced by certain pathogens are a critical means of understanding the role of individual pathogens in the disease process. Controlled inoculation studies limit the effect of other biotic agents, including saprophytes, which could mask the effect of a specific pathogen. Additionally, yield losses could be attributed to the wrong microorganism if field evaluation is performed at a late stage of the grain maturation process.

The results of this work suggest that *B. glumae* is a significant biotic threat to panicle sterility in rice fields. Greenhouse inoculation assays showed that *B. glumae* isolates caused higher levels of sterility and greater inhibition of grain filling than *B. gladioli* isolates. On the other hand, *B. gladioli* reduced grain quality due to the high level of dark brown lesions on affected seed. Panicles infected with *B. gladioli* rarely showed grain discoloration (i.e., loss of color in the palea and lemma) or abortions, symptoms more commonly produced by *B. glumae*. Our results demonstrate that, although both species of *Burkholderia* can be found on rice seed, one is more detrimental to grain yield and the other is more relevant for grain quality traits. Although results presented here demonstrate that *B. glumae* has a tremendous potential for limiting rice production under favorable conditions, we urge caution in assigning rice yield losses to just *B. glumae*, at least in those fields where disease incidence data at the flowering stage is absent. Furthermore, although *B. gladioli* is not as aggressive as *B. glumae* in terms of damage, yield reduction, or infection speed, this work demonstrates the substantial impact that *B. gladioli*-related BPB symptoms may have on grain quality, which can translate into large economic losses for the farmers due to the lower price of stained grains.

The phenotypic differences between *B. glumae* and *B. gladioli* infections may suggest a difference in infection strategies between the two pathogens; whereas *B. glumae* is much more aggressive in halting the grain filling process, indicative of a mechanism of inhibiting seed formation, *B. gladioli* appears

limited to colonizing the panicle without interfering with pollination, fertilization, or other seed-formation processes. However, the possibility that this bacterium could impact fertilization downstream should not be ignored. *B. gladioli* may colonize flower tissue and induce a stronger host reaction, as shown by the brown coloration of the grains. Brown coloration observed on infected palea and lemma by *B. gladioli* but less evident in *B. glumae* is intriguing and needs further studies to identify the cellular process involved in this plant response. We also found that co-invasion of both bacteria on the same tissue was relatively rare, which could suggest that disease does not normally occur as a complex of the two species, although this finding could also be related to the difference in geographical distribution of these two species in Colombia. Additional studies are needed to establish the time point of panicle infection of each species under field conditions, and to measure their survival capacity on infected panicles when they are co-inoculated under controlled conditions. Those results will give relevant information on potential growth inhibition behavior in any of these bacteria.

In analyzing just a few selected genomic differences between the four sequenced *B. glumae* and two sequenced *B. gladioli* strains, we found that the two species have highly conserved genes for the characterized virulence factors involved in toxin and lipase production and quorum sensing but have highly distinct repertoires of genes for certain families of transcriptional regulators, methyl-accepting chemotaxis receptors, major facilitator superfamily proteins, and T6SS components. Although *B. glumae* encodes fewer genes than *B. gladioli*, genes for several potential virulence factors (T6SS-5 clade secretion genes, HrpW, and Eop3 T3SS effectors) are specific to, or highly divergent in, this species. The categories of transcriptional regulators and membrane proteins categorized here represent some of the largest functional gene families in prokaryotes, with diverse, mostly uncharacterized roles in sensing and responding to a vast range of environmental stimuli (14,19,41); therefore, any differential roles in the lifestyles of *B. glumae* and *B. gladioli* may prove difficult to define. Many other virulence factors identified in other *Burkholderia* pathogens, such as type II and IV secretion systems, siderophores, lipopolysaccharides, and adhesins, were not specifically analyzed in this work; small single-site differences could also play a large role in determining phenotype. The continually increasing availability of sequence from the rice-pathogenic *Burkholderia* spp. will lay a rich groundwork for future in-depth comparative analysis, high-throughput genetic manipulation, and

TABLE 4. Presence and sequence homology of known and potential virulence factors among *Burkholderia glumae* and *B. gladioli* genome sequences

Gene category, product description	BGR1 locus	Nucleotide identity of match (%) in <i>B. glumae</i> , <i>B. gladioli</i> draft genomes ^y				BSR3 locus (identity with BGR1 [%])
		3252-8	Au6208	LMG2196	3848s-5	
Type III effector genes						
XopAL1/Eop3	bglu_2g03280	99	99	100	...	...
HrpK1	bglu_2g02530	99	100	100	92	bgla_2g02920 (92)
HrpW	bglu_2g02490	99	99	100	...	...
Hpr kinase	bglu_2g04890	99	99	100	86	bgla_2g06400 (85)
Awr-family protein	bglu_2g02250	99	...	100	80	bgla_2g16980 (64)
YgbK domain protein	...	...	...	...	100 ^z	bgla_1g16070
Toxoflavin biosynthesis genes and regulators						
ToxR	bglu_2g06390	99	99	99	95	bgla_1g04560 (97)
ToxA	bglu_2g06400	99	99	99	96	bgla_1g04570 (97)
ToxB	bglu_2g06410	99	100	100	96	bgla_1g04580 (98)
ToxC	bglu_2g06420	99	99	100	95	bgla_1g04590 (97)
ToxD	bglu_2g06430	100	99	100	95	bgla_1g04600 (97)
ToxE	bglu_2g06440	99	99	100	93	bgla_1g04610 (96)
TofR	bglu_2g14470	100	99	99	90	bgla_2g11070 (92)
Lipase						
LipA	bglu_2g07730	99	99	100	87	Bgla_2g00010 (87)

^y Although gene queries usually matched the draft assemblies with 100% sequence coverage, the presence of a complete open reading frame could not be confirmed in every genome due to gaps in some draft assemblies.

^z Nucleotide identity to the predicted gene in *B. gladioli* BSR3.

in planta gene expression studies to help determine which genes are likely to play a role in infection.

It is still not clear what relative losses are caused by *B. gladioli* in field conditions or, potentially, in synergistic infections with *B. glumae*, although *B. gladioli* tends to be overlooked as the less-frequently isolated of the two species. However, it is possible that *B. gladioli* will gain prevalence in the future as a consequence of unstable climate conditions and rice crop intensification, as is already suspected to be happening with *B. glumae* (22,24). For this reason, studies on identification of potential sources of tolerance to this bacterium in rice germplasm will be crucial for breeding of resistant cultivars. We have established a standardized greenhouse protocol for inoculation and evaluation of *B. glumae* and *B. gladioli* isolates on rice germplasm, and have developed a list of several candidate genes of interest that differentiate the two species. Although the sequenced strain was not used in the pathogenicity studies reported here, all strains tested from Colombia showed indistinguishable pathogenicity patterns and morphology.

The inoculation method developed here is now being used to screen more genotypes for sources of resistance or tolerance, to determine whether the phenotypic patterns we observed are consistent among other susceptible varieties, and to analyze genetic variants of *Burkholderia* spp. for yield loss phenotypes.

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