The In Vitro and In Planta Interspecies Interactions Among Rice-Pathogenic *Burkholderia* Species

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Abstract

Burkholderia glumae, *B. plantarii*, and *B. gladioli* are responsible for serious diseases in rice crops and co-occurrence among them has been reported. In this study, in vitro assays revealed antagonistic activity among these organisms, with *B. gladioli* demonstrating strong inhibition of *B. glumae* and *B. plantarii*. Strains of *B. glumae* and *B. plantarii* that express green fluorescent protein were constructed and used for cocultivation assays with *B. gladioli*, which confirmed the strong inhibitory activity of *B. gladioli*. Cell-free supernatants from each species were tested against cultures of counterpart species to evaluate the potential to inhibit bacterial growth. To investigate the inhibitory activity of *B. gladioli* on *B. glumae* and *B. plantarii* in rice, rice plant assays were performed and quantitative PCR

(qPCR) assays were developed for in planta bacterial quantification. The results indicated that coinoculation with *B. gladioli* leads to significantly reduced disease severity and colonization of rice tissues compared with single inoculation with *B. glumae* or *B. plantarii*. This study demonstrates the interactions among three rice-pathogenic *Burkholderia* species and strong antagonistic activity of *B. gladioli* in vitro and in planta. The qPCR assays developed here could be applied for accurate quantification of these organisms from in planta samples in future studies.

Keywords: *Burkholderia glumae*, *Burkholderia gladioli*, *Burkholderia plantarii*, interaction, qPCR

Rice (*Oryza sativa* L.) is among the main cereal crops cultivated worldwide, representing the primary food source for almost 50% of the human population (Awika 2011). Protection of rice cultivation is crucial to maintain a continuous supply of this grain and sustain the growing human population (Walter et al. 2016). Currently, bacterial diseases are among the major threats to rice cultivation and climate change predictions indicate that they are expected to represent a more serious threat (Schaad 2008).

The genus *Burkholderia* encompasses a diverse group of gram-negative species, characterized mainly by large multireplicon genomes with high genetic plasticity; this allows them to occupy diverse ecological niches and carry out several key roles within their respective ecosystems (Mannaa et al. 2019; Seo et al. 2015). Three *Burkholderia* species have been reported to be pathogenic to rice. Most importantly, *B. glumae* is responsible for panicle blight and seedling and grain rot (Goto and Ohata 1956; Ham et al. 2011); this organism was first reported in Japan and has emerged in several other locations as a major threat to rice cultivation. The second main rice phytopathogen within the *Burkholderia* group is *B. plantarii*, a reported cause of seedling blight (Azegami et al. 1987). Both *B. glumae*

and *B. plantarii* are responsible for devastating losses in rice production, in seedlings in nursery boxes, and in the field; this is primarily attributable to the production of phytotoxins toxoflavin and tropolone, respectively (Hikichi et al. 1995; Maeda et al. 2006). The third important rice pathogen is *B. gladioli*, which has been reported to cause rot symptoms in several vegetable crops (Hildebrand et al. 1973; Lincoln et al. 1991). Rice blight and grain rot symptoms, resembling those caused by *B. glumae*, have also been attributed to *B. gladioli* and this pathogen has been isolated from infected rice leaves and grains (Kato et al. 1992; Ura et al. 2006).

Epidemiological and pathogenesis studies on rice-pathogenic *Burkholderia* species have usually focused on single-species infections, although multiple reports have indicated some evidence of possible interactions with other microbes. For example, the cooperative relationship between *B. glumae* and the fungal pathogen *Fusarium graminearum* indicates a unique strategy by which both etiological agents cause more severe disease in rice when combined than when alone (Jung et al. 2018). Furthermore, few studies have reported the coexistence of *B. glumae* and *B. gladioli* in infected rice fields (Fory et al. 2014; Nandakumar et al. 2009). Other studies have indicated a negative correlation between *B. gladioli* and the other two *Burkholderia* species in rice (Miyagawa 2000; Riera-Ruiz et al. 2018).

Microbial communities, particularly those sharing the same habitat, are usually in continuous dynamic interaction with one another in a variety of ways. Classification of the possible interaction patterns (i.e., mutualism, commensalism, parasitism, amensalism, and competition) is usually based on the negative or positive impact on the involved species (Faust and Raes 2012). Interaction studies typically focus on antagonism or mutualism among different groups of microbes, and only a few studies have considered interactions among species within the same group of bacteria sharing the same host. In light of this fact, investigation of the possible interactions among these three rice-pathogenic *Burkholderia* species could improve understanding of the disease development process in rice.

In this study, we aimed to investigate the interaction among the three rice-pathogenic *Burkholderia* species (*B. glumae* BGR1, *B. gladioli* KACC18962, and *B. plantarii* KACC18965) in vitro and in planta. To facilitate studying their interactions in planta, we aimed to develop a species-specific quantitative PCR (qPCR) assay for

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quantification of these *Burkholderia* spp. from rice. Evaluating the interactions among these *Burkholderia* species will contribute to the overall understanding of their respective disease-causing mechanisms and may provide options for their management. Moreover, the qPCR assay developed for in planta quantification of rice-pathogenic *Burkholderia* may be useful for studying their behaviors within the host and for future studies on their interactions with other microbes or environmental conditions.

Materials and Methods

Detection of naturally occurring coinfection between the three rice-pathogenic bacterial species. In a survey conducted in rice fields in Korea, 77 symptomatic rice panicles showing typical bacterial blight and rot symptoms were collected and used for molecular detection of the rice-pathogenic *Burkholderia* species, *B. glumae*, *B. gladioli*, and *B. plantarii*. The primers used for molecular detection in this survey are shown in Supplementary Table S1. Bacterial DNA was extracted from symptomatic rice panicles as described by Nandakumar et al. (2009). PCR assays were conducted as described in previous studies (Maeda et al. 2006; Nandakumar et al. 2009; Noh et al. 2012).

Bacterial strains and mutants used in this study. All bacterial strains used in this study, including the three *Burkholderia* rice pathogens, *B. glumae* BGR1, *B. gladioli* KACC18962, and *B. plantarii* KACC18965 and their respective sources, are listed in Table 1. All strains were initially grown on Luria-Bertani (LB; Difco) agar, from which a single colony was inoculated into LB broth and incubated with shaking (200 rpm) at 37°C (*Escherichia coli* and *B. glumae* BGR1) or 28°C (*B. gladioli* KACC18962 and *B. plantarii* KACC18965) based on optimal growth temperature. Optical density of the bacterial cultures at 600 nm (OD₆₀₀) was evaluated using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

To facilitate studying the effects of cocultivation of *B. gladioli* KACC18962 with *B. glumae* BGR1 and *B. plantarii* KACC18965, green fluorescent protein (GFP)-expressing *B. glumae* and *B. plantarii* strains were generated. Standard methods for DNA manipulation, cloning, and gel electrophoresis were used (Sambrook et al. 1989). Briefly, to prepare the GFP expression vector (pBBR1P2::gfp), the *recA* promoter fragment and pBBR1MCS-2 (Masahiro et al. 1984) were amplified, then digested using *Kpn*I and *Hind*III restriction enzymes (New England Biolabs, Ipswich, MA) for 2 h at 37°C, and ligated with T4 DNA ligase (New England Biolabs) at 4°C overnight. The *gfp* open reading frame was amplified from the commercial plasmid pUBN-GFP-DEST (Addgene, Watertown, MA) using the primer pair, GFP_HindIII_F and GFP_BamHI_R. The amplified *gfp* fragment and pBBR1P2 plasmid were ligated such that *gfp* was flanked by the 3' region of the *recA* promoter sequence.

Recombinant plasmids were transformed into *E. coli* DH5 and then selected for growth on kanamycin (50 µg/ml). *B. glumae* BGR1 was transformed by conjugation with *E. coli* S17-1, carrying pBBR1P2::gfp. Successfully transformed *B. glumae* BGR1 colonies were selected on LB agar containing kanamycin (50 µg/ml) and rifampicin (100 µg/ml).

Transformation of *B. plantarii* KACC18965 was performed by electroporation after electrocompetent cells were prepared following Anthony's protocol (Smith and Iglewski 1989). Briefly, *B. plantarii* KACC18965 was cultured at 28°C until an OD₆₀₀ of 0.4 was reached and then the cells were pelleted by centrifugation at 6,000 × g for 5 min. The cell pellet was washed twice using 300 mM of sucrose and then resuspended in 100 µl of 300 mM sucrose. The cell suspension was cooled on ice for 30 min, then 300 ng of pBBR1P2::gfp was mixed with 50 µl of cells. Electroporation was performed using ice-chilled 0.2-cm gap cuvettes (Bio-Rad, Hercules, CA) at 2.5 kV using a MicroPulser electroporator (Bio-Rad). Electroporated cells were mixed directly with 500 µl of fresh LB broth and incubated for 4 h at 28°C. After incubation, bacterial cells were selected on LB agar containing kanamycin (50 µg/ml). The transformed strains were confirmed molecularly and phenotypically by PCR and fluorescence microscopy (pE-300white; CoolLED, San Francisco, CA). All primers and plasmids used are listed in Supplementary Table S2.

In vitro interactions among rice-pathogenic *Burkholderia* spp. To test the antagonistic effect among the three *Burkholderia* species, double-layer LB agar plates were used as follows: bacterial cultures (OD₆₀₀ = 0.5) were combined with LB containing 0.8% (wt/vol) agar at a ratio of 1:10 and were poured over a primary layer of 10 ml of 1.5% (wt/vol) LB agar. Sterilized paper discs (ADVANTEC, Tokyo, Japan) were placed on the top of the inoculated agar, and 10 µl of bacterial culture (OD₆₀₀ = 0.5) of the counterpart species was added onto the paper discs. Plates were incubated at 28°C and another set of plates were incubated at 37°C for 3 days; the inhibition zone, indicating antibacterial activity, was observed.

Based on the results of the disc diffusion assays, cocultivation assays were performed. The experiments were modified from the method described by Hachani et al. (2013). Briefly, when *B. glumae* BGR1/gfp was used as a prey organism, *B. gladioli* KACC18962 or *B. plantarii* KACC18965 was used as the counterpart organism. When *B. plantarii* KACC18965/gfp was used as prey, *B. gladioli* KACC18962 or *B. glumae* BGR1 was used as the counterpart. Prey and counterpart (OD₆₀₀ = 0.5; equivalent to colony-forming unit [CFU] values of $2.1 \times 10^8 \pm 4.3$, $6.5 \times 10^8 \pm 2.3$, and $7.1 \times 10^8 \pm 2.5$ for *B. glumae*, *B. gladioli*, and *B. plantarii*, respectively) were mixed at a ratio of 1:1, and 100 µl was spotted on LB agar and incubated at 28 or 37°C for 20 h. The bacterial cells grown on LB agar after incubation were resuspended in 1 ml of LB broth and assessed

Table 1. Bacterial strains and plasmids used in this study

Name ^z	Description	Source
Bacterial strain		
<i>Escherichia coli</i>		
DH5α λpir	Used for plasmid replication	Laboratory collection
S17-1 λpir	Used as donor cell for conjugation	Laboratory collection
<i>Burkholderia glumae</i>		
BGR1	<i>B. glumae</i> isolate from rice, wild type, rifampicin resistant	Kim et al. (2004)
BGR1/gfp	BGR1, transformed with pBBR1P2::gfp	This study
<i>B. gladioli</i>		
KACC18962	<i>B. gladioli</i> isolate from rice, wild type	KACC
<i>B. plantarii</i>		
KACC18965	<i>B. plantarii</i> isolate from rice, wild type	KACC
KACC18965/gfp	KACC18965, transformed with pBBR1P2::gfp	This study
Plasmid		
pBBR1MCS-2	Broad-host-range vector, kanamycin resistant	Kovach et al. (1995)
pBBR1P2	pBBR1MCS-2::recA promoter region	This study
pBBR1P2::gfp	pBBR1P2::gfp gene	This study
pUBN-GFP-DEST	Template for <i>gfp</i> gene amplification	Addgene

^z KACC = Korean Agricultural Culture Collection (<http://genebank.rda.go.kr>) and GFP = green fluorescent protein.

in two ways. First, 100 μ l was transferred to a cell culture plate (SPL Life Sciences, Pocheon, Korea) and fluorescence intensity was measured with a Mithras LB 940 multimode microplate reader (Berthold, Thoiry, France). Relative fluorescence intensity (RFI) was calculated in comparison with a media-only control. For the second analysis, cell density was adjusted to an OD₆₀₀ of 0.5, serially diluted, and spotted onto LB agar containing kanamycin (50 μ g/ml); plates were incubated at 37 or 28°C and CFUs were quantified.

Cell-free supernatants (CFSs) were tested to investigate their potential inhibitory activity. Cultures at late-log to stationary phase were centrifuged at 6,000 $\times$ g for 5 min; cell pellets were discarded and the supernatant was filtered using a 0.2- μ m Minisart syringe filter (Sartorius, Göttingen, Germany). The CFSs were then added to 2 ml of LB broth at a final concentration of 0.1, 1, or 10% (vol/vol), respectively. LB broth without CFS was used as a control. The counterpart *Burkholderia* species were cultured until an OD₆₀₀ of 0.5 was reached and 200 μ l of cell culture was added to the CFS-LB broth dilutions. After incubation for 8 h, growth of the counterpart species was estimated by measurement of the OD at 600 nm.

Development of qPCR for quantification of rice-pathogenic *Burkholderia* spp. To develop an efficient method for specific in planta quantification, we used novel primer pairs (Supplementary Table S3) designed with a comparative genomic approach (Lee et al. 2018). The primer pairs (15 for *B. glumae*, 13 for *B. gladioli*, two for *B. plantarii*) were screened to determine efficient and species-specific pairs for application in qPCR. Specificity to target species was assessed by PCR using genomic DNA (gDNA) from target and nontarget species templates. Bacterial gDNA from the three *Burkholderia* species and other nontarget species (*Pantoea* sp., *Bacillus* sp., *Pseudomonas* sp., and *E. coli* DH5 α) was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI), following the manufacturer's instructions. PCR was performed using *Taq* polymerase (TaKaRa, Kusatsushi, Japan) and the

following protocol: 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at annealing temperature (60°C), 1 min/kb at 72°C, and 5 min at 72°C.

To further test the specificity of the selected primer pairs for producing single amplicons and their applicability in these qPCR assays, melting curve analyses were performed using target gDNA and the target standard product, which included the target sequence of the primer pair under evaluation. A temperature gradient of 1°C/s from 65 to 95°C was employed after running the qPCR reaction. PCR products were visualized by electrophoresis using 2% (wt/vol) agarose gel for confirmation.

In planta interaction among rice-pathogenic *Burkholderia* spp. To confirm the observed in vitro inhibitory effect of *B. gladioli* KACC18962 against *B. glumae* BGR1 and *B. plantarii* KACC189, in planta rice (*O. sativa* L. 'Saeilmi') assays were performed at the vegetative and seedling stages, respectively. For the rice stem assay, rice plants were grown under greenhouse conditions until the vegetative stage. Stems were inoculated by stabbing (approximately 0.2 cm deep) with a single bacterial colony, using a sterilized toothpick. For coinfections, single colonies of each species were inoculated in the same stab. Infected stems were collected 11 days postinoculation for evaluation of disease severity and quantification of the bacteria in infected tissues. For the seedling assay, rice seeds were first surface sterilized in 70% ethanol for 10 min, followed by 1% hypochlorite solution for 10 min; they were then vigorously rinsed five times in sterilized distilled water (SDW). Surface-sterilized seeds were inoculated by immersion in bacterial suspensions (at OD₆₀₀ = 0.8; equivalent to CFU values of $3.1 \times 10^8 \pm 5.7$, $1.0 \times 10^9 \pm 2.7$, and $1.3 \times 10^9 \pm 2.1$ for *B. glumae*, *B. gladioli*, and *B. plantarii*, respectively) singly or in a mixture of 1:1 of two species in the dark for 48 h at 28°C (as the optimum temperature for rice seedling growth). Seeds immersed in SDW served as the negative control. Germinated seeds were then transferred to plant culture tubes (SPL Life Sciences, Pocheon, Korea) containing 15 ml of SDW and incubated in a humid growth chamber at 28°C using a 12-h day/nighttime light system. Seven days postinoculation, the seedling shoot length was measured as an indication of disease severity and the bacterial load inside the seedlings was quantified.

Fig. 1. Disc diffusion double-layer agar to investigate in vitro interactions among *Burkholderia glumae* BGR1, *B. gladioli* KACC18962, and *B. plantarii* KACC18965. Experiments were performed at **A**, 28°C or **B**, 37°C. The autoclaved paper discs were soaked in bacterial suspension and placed on the top agar seeded with counterpart bacterial cells. Inhibitory activity was detected as a clear halo zone surrounding the paper disc. Observed activities are schematized on the squares on the right as follows: +++ = strong activity with obvious halo zone, ++ = weak activity with obvious halo zone, and + = weak activity with faint halo zone. Dashes indicate no activity.

Fig. 2. Relative fluorescence intensity (RFI) of transformants after cocultivation. Fluorescence microscopy images of **A**, *Burkholderia glumae* BGR1/gfp and **B**, *B. plantarii* KACC18965/gfp. Experiments were conducted at 28 or 37°C, and the effect of cocultivation on counterpart species was estimated by evaluation of the RFI of green fluorescent protein (GFP) represented as follows: (fluorescence intensity/OD₆₀₀ $\times$ 1,000). The RFI of **C**, *B. glumae* BGR1/gfp and **D**, *B. plantarii* KACC18965/gfp are shown. Compared with the control (sole culture), all treatments at 28 and 37°C showed a significant difference at $P < 0.001$, except for the effect of *B. plantarii* KACC18965 on *B. glumae* BGR1/gfp at 37°C ($*P < 0.05$). OD₆₀₀ = optical density at 600 nm.

Application of qPCR for in planta quantification of rice-pathogenic *Burkholderia* spp. DNA was extracted from treated rice stems and seedlings as follows: 100 mg (collected from the stem assay by avoiding the infection cut with approximately 3 mm and from the shoots of the seedlings in the seedling assay) was transferred into 2-ml Safe-Lock tubes (Eppendorf, Hamburg, Germany) containing 5-mm stainless steel beads (Qiagen, Hilden, Germany) and deep frozen at -80°C . A Tissue Lyser II (Qiagen) was used for sample grinding at a frequency of 20 per second for 30 s. During all steps, samples were kept frozen. After grinding, DNA extraction was performed following the classic cetyltrimethylammonium bromide (CTAB)-based protocol with modifications (Murray and Thompson 1980). Briefly, 7 ml of CTAB buffer (100 mM of Tris-HCl, 1.2 M of NaCl, 20 mM of EDTA, 2% CTAB, and 0.2% β -mercaptoethanol) was added to the ground samples in a 15-ml conical tube and vortexed vigorously for 20 s. The mixture was heated in a water bath at 65°C for 1 h, then 6 ml of 24:1 (vol/vol) chloroform/isoamyl alcohol solution (CI) was added and mixed thoroughly by inversion. The mixture was then centrifuged at $13,000 \times g$ for 8 min and 6.5 ml of the supernatant was again combined with 6.5 ml of 24:1 (vol/vol) CI and mixed and centrifuged as before. The supernatant (5.5 ml) was combined with 3.3 ml of isopropanol containing RNase A (50 $\mu\text{g/ml}$ final concentration) and inverted 10 times. The mixture was kept at -20°C for 12 h for precipitation, then centrifuged at $13,000 \times g$ for 15 min. The

supernatant was discarded, and DNA pellets were washed with 1.25 ml of 70% (vol/vol) ethanol and eluted by centrifugation at $13,000 \times g$ for 5 min. DNA pellets were air-dried and resuspended in 1 ml of nuclease-free water (Ambion, Chipping Norton, UK). Extracted DNA quality and concentration was evaluated using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and 0.8% (wt/vol) agarose gel electrophoresis.

qPCR was performed in a total volume of 20 μl , containing 10 μl of TOPrea qPCR 2 $\times$ PreMix (SYBR Green with low ROX) (Enzygnomics, Daejeon, Korea), 1 μl of template DNA, 1 μl of each primer (10 μM), and nuclease-free water. PCR was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) and data were analyzed using Rotor-Gene Q software version 2.3.1 (Qiagen, Hilden, Germany). The reaction cycle was as follows: initial denaturation for 10 min at 95°C , followed by 35 cycles of 10 s at 95°C , 15 s at 60°C , and 20 s at 72°C . All samples were measured in triplicate and the average of the three replicates was calculated as a single cycle threshold (C_T) value. No template controls were included in each run. The three selected primer pairs were used for amplification of the target *Burkholderia* species (Supplementary Table S2).

Serially diluted standard PCR products for each primer pair were used to generate standard curves to facilitate quantification (Morrison et al. 1999). Standard PCR product copy numbers were calculated (<https://cels.uri.edu/gsc/cndna.html>) to be in the range of 3×10^8

Fig. 3. Bacterial populations of the transformants after cocultivation with counterpart species. Cocultivation was performed at two different temperature conditions, 28 or 37°C . Ten-fold serial dilution series, from 10^{-3} to 10^{-7} , of bacterial resuspension (cocultivated or sole culture control) were spotted on Luria-Bertani (LB) plates containing kanamycin (50 $\mu\text{g/ml}$) and incubated at **A**, 37°C for *Burkholderia glumae* BGR1/gfp and **B**, 28°C for *B. plantarii* KACC18965/gfp. LB media were used as the negative control. After incubation, colony-forming units (CFUs) per milliliter were calculated and are presented here. Compared with the sole culture control, all treatments at 28 and 37°C showed a significant difference at $P < 0.001$, except for the population of *B. glumae* BGR1/gfp cocultivation with *B. plantarii* KACC18965 at 37°C . ns = not significant.

to 3×10^3 copies/ μ l. The C_T values were plotted relative to the corresponding serial dilution series in a linear regression model that was then used for the absolute quantification of the three target *Burkholderia* species.

Statistical analysis. All experiments were conducted twice with at least three replicates and statistical analyses were performed using Statistical Analysis Systems software (SAS Institute, Cary, NC). Analysis of variance was conducted using generalized linear model

procedures, and comparisons between means were performed using the least significant difference test at $P < 0.05$.

Results

Detection of coinfection among the rice-pathogenic *Burkholderia* species. The survey conducted in Korean rice fields resulted in collection of 77 symptomatic rice panicles showing typical bacterial blight and rot symptoms. The collected samples were

Fig. 4. Effect of cell-free supernatant (CFS) on bacterial populations. **A**, Effect of CFSs from *Burkholderia glumae* BGR1 on the population of *B. gladioli* KACC18962 and *B. plantarii* KACC18965. **B**, Effect of CFS from *B. gladioli* KACC18962 on the population of *B. glumae* BGR1 and *B. plantarii* KACC18965. **C**, Effect of CFS from *B. plantarii* KACC18965 on the population of *B. gladioli* KACC18962 and *B. glumae* BGR1. CFS from cultures incubated at 28 and 37°C was diluted in Luria-Bertani (LB) broth to a final concentration of 0.1, 1, and 10% (vol/vol); LB broth alone was used as a control. Counterpart species were inoculated in LB broth containing each CFS and cultured with shaking (200 rpm) for 8 h; growth was estimated by optical density at 600 nm (OD_{600}). Different letters on the error bars (standard deviation, $n = 3$) indicate a significant difference according to the least significant difference test at $P < 0.05$.

used for molecular detection of the rice-pathogenic *Burkholderia* species, *B. glumae*, *B. gladioli*, and *B. plantarii*. Among the collected samples, 51 were positive in PCR assays specific for *B. glumae*, three were positive for *B. gladioli*, and five were positive for *B. plantarii*. More interestingly, coinfection with *B. glumae* and *B. gladioli* was confirmed in two samples and coinfection with *B. glumae* and *B. plantarii* was confirmed in three samples (Supplementary Fig. S1).

In vitro interactions among rice-pathogenic *Burkholderia* species. A double-layer LB agar plate disc diffusion method was used to test antagonistic activity between the three *Burkholderia* species. In this assay, *B. gladioli* KACC18962 exhibited strong inhibitory activity against both *B. glumae* BGR1 and *B. plantarii* KACC18965, demonstrated by the presence of a clear halo around the *B. gladioli* KACC18962 disc when incubated with either species at 28°C (Fig. 1A). *B. plantarii* KACC18965 also demonstrated mild inhibitory activity against *B. glumae* BGR1 at 28°C. However, at 37°C, inhibitory activity was only observed in *B. glumae* BGR1 and *B. gladioli* KACC18962, both acting against *B. plantarii* KACC18965 (Fig. 1B). The incubation temperature was shown to affect antagonistic activity, as the optimum temperature was different among the strains used.

To further investigate these observed antagonistic effects, cocultivation experiments were conducted using *B. gladioli* KACC18962 and GFP-expressing strains of *B. glumae* BGR1 and *B. plantarii* KACC18965. The GFP-expressing strains produced a green fluorescence that can be visualized (Fig. 2A and B) and measured to estimate the RFI. The RFI of both *B. glumae* BGR1/gfp and *B.*

plantarii KACC18965/gfp was significantly lower ($P < 0.001$) when cocultured with other species compared with that of sole culture at both 28 and 37°C. The RFI of *B. glumae* BGR1/gfp cocultured with *B. plantarii* KACC18965 at 37°C, which was significantly less than the sole culture control at $P < 0.05$ (Fig. 2C and D). In particular, cocultivation with *B. gladioli* KACC18962 resulted in a drastic reduction in the RFI of the other two species, where 90% *B. glumae* BGR1 growth was inhibited at both incubation temperatures. These results indicate that *B. gladioli* KACC18962 has the strongest antagonistic activity against the other two species. The stability of the GFP construct in the cocultivation assay without antibiotic selection was confirmed, as no significant difference was observed in RFI or antagonistic activity in media with or without antibiotic selection and in mutants containing empty vectors (Supplementary Fig. S2).

The strong inhibitory activity of *B. gladioli* KACC18962 against *B. glumae* BGR1/gfp and *B. plantarii* KACC18965/gfp was further confirmed by assessment of the viable CFUs of both strains compared with their sole culture controls, which almost disappeared by cocultivation with *B. gladioli* KACC18962 (Fig. 3A and B). Inhibitory activity was also observed between *B. glumae* BGR1 and *B. plantarii* KACC18965 at both incubation temperatures except for the effect of *B. plantarii* KACC18965 on *B. glumae* BGR1/gfp at 37°C, which was not significantly different compared with the sole culture control (Fig. 3A).

To investigate the potential of inhibitory extracellular metabolite production by the three species tested, the impact on growth of CFS derived from cultures incubated at 28 and 37°C was assessed.

Fig. 5. Standard curves for the real-time PCR of serially diluted bacterial cultures. **A**, Standard curve of *Burkholderia glumae* BGR1 cultures using Bglu. **B**, Standard curve of *B. gladioli* KACC18962 cultures using Bgla. **C**, Standard curve of *B. plantarii* KACC18965 cultures using Bpln. The bacterial cultures were serially diluted ranging from 10^4 to 10^8 colony-forming units (CFUs)/ml and used for standard amplification. For each primer pair, C_T measurements were plotted against their actual CFU. Parameters of each standard curve are noted as follows: r^2 = coefficient of determination, E = amplification efficiency, and M = the slope of regression.

CFS from *B. glumae* BGR1 (28 or 37°C) had no significant effect on *B. gladioli* KACC18962 at all concentrations (0.1 to 10%). However, 1 and 10% dilutions of *B. glumae* BGR1 CFS collected at 37°C showed significant inhibitory activity against *B. plantarii*

KACC18965 (Fig. 4A). All concentrations of *B. gladioli* KACC18962 CFS from 37°C cultures showed a significant effect against *B. glumae* BGR1, as did 1 and 10% from 28°C cultures. In addition, all concentrations of *B. gladioli* KACC18962 CFS from

Fig. 6. In planta assays to detect interactions among rice-pathogenic *Burkholderia* species. **A**, The observed lesions on rice stems 11 days postinoculation (DPI), after inoculation with single colonies from *B. glumae* BGR1 and *B. gladioli* KACC18962 by stabbing into rice stems, separately or in combination (1:1). **B**, Disease severity on rice stems expressed as the length of diseased lesion (in centimeters). Different letters on the error bars (standard deviation, $n = 4$) indicate significant differences between treatments, according to the least significant difference (LSD) test at $P < 0.05$. **C**, The observed symptoms on rice seedlings at 7 DPI, after inoculation by immersion in culture suspensions ($OD_{600} = 0.8$) of *B. gladioli* KACC18962 and *B. plantarii* KACC18965 (1:1 combination). **D**, Disease severity on the inoculated seedlings, as estimated by the length of shoots (in centimeters). Different letters on the error bars (standard deviation, $n = 12$) indicate significant differences between treatments according to the LSD test at $P < 0.05$. Sterilized distilled water (SDW) was used as a negative control in both in planta assays. OD_{600} = optical density at 600 nm.

37°C cultures showed a significant effect against *B. plantarii* KACC18965, whereas this was only observed with the 10% dilution from 28°C cultures (Fig. 4B). Finally, *B. plantarii* KACC18965 CFS showed a significant effect against *B. gladioli* KACC18962 (1 and 10%) and *B. glumae* BGR1 (10%) at both temperatures (Fig. 4C). The results showed that the CFS of each strain against its counterpart has an antagonistic effect, with the only exception being *B. glumae* against *B. gladioli* at both temperatures. This indicates that at least *B. plantarii* may have the capacity to control *B. gladioli* when they are tested under similar conditions.

Selected primer pairs are species specific and applicable in qPCR for rice-pathogenic *Burkholderia* spp. One primer pair was selected for each species based on screening results from the conventional PCR assay as follows: Bglu8 for *B. glumae* BGR1, Bglu10 for *B. gladioli* KACC18962, and Bpln2 for *B. plantarii* KACC18965 (Supplementary Table S3). Species specificity was confirmed by testing with other nontarget species, where clear, visible bands were only observed with the target species at the expected target product sizes (Supplementary Fig. S3). The applicability of the selected primers to qPCR application and production of single amplification products was confirmed by the melting curve analysis on bacterial gDNA and standard products, which resulted in single dominant peaks at 86.7, 91.2, and 90.2°C for the Bglu8, Bglu10, and Bpln2 primer pairs, respectively (Fig. 5; Supplementary Fig. S4 and S5).

Coinfection with *B. gladioli* affects disease severity and colonization of rice by *B. glumae* and *B. plantarii*. In planta assays were conducted to confirm the strong inhibitory activity of *B. gladioli* KACC18962 against *B. glumae* BGR1 and *B. plantarii* KACC18965 observed in the in vitro assays. Rice stems coinoculated with *B. glumae* BGR1 and *B. gladioli* KACC18962 demonstrated a visible reduction in symptomatic lesions compared with sole inoculation with *B. glumae* BGR1 (Fig. 6A), and these lesions were confirmed to be shorter in length (in centimeters) when measured (Fig. 6B). Because *B. plantarii* is more often associated with rice seedling rot, a seedling assay was conducted to test the effect of coinfection with *B. gladioli*. Similar to the previous assay results, coinoculation with a mixture of *B. plantarii* KACC18965 and *B. gladioli* KACC18962 resulted in a visible reduction in disease symptoms compared with seedlings inoculated with a sole culture of *B. plantarii* KACC18965 (Fig. 6C). Equally, using seedling shoot length as an indicator of disease development, inoculation with either strain in sole or mixed culture resulted in a significant reduction compared with a SDW control. Moreover, it was confirmed that the coinoculation of *B. plantarii* KACC18965 with *B. gladioli* KACC18962 resulted in reduced virulence of *B. plantarii*, as significantly longer shoots were observed in the coinoculated seedlings compared with sole inoculation with *B. plantarii* KACC18965 (Fig. 6D). In both rice assays, inoculation with *B. gladioli* KACC18962 alone resulted in weak disease and mild symptoms.

To test the effect of coinoculation on bacterial ability to colonize the tissues of rice plants, infected tissues were analyzed using qPCR. A standard curve was first generated by serially diluting standard

PCR products from the three *Burkholderia* species and plotting the resulting reaction C_T values; a regression model was used for the absolute quantification of the three *Burkholderia* species in rice tissues (Supplementary Fig. S4). The standard curves of the PCR products were also compared with standard curves generated using serially diluted bacteria cultures with known CFUs per milliliter (estimated by standard plate count on LB agar) for confirmation (Fig. 5). Coinoculation of *B. glumae* BGR1 with *B. gladioli* KACC18962 resulted in a significant reduction in the population of *B. glumae* BGR1 compared with sole inoculation in the rice stem assay ($P < 0.01$; Table 2). Similarly, in the seedling assay, coinoculation of *B. plantarii* KACC18965 with *B. gladioli* KACC18962 resulted in a significant reduction in the population of *B. plantarii* KACC18965 compared with sole inoculation ($P < 0.05$; Table 2). In both in planta assays, there was no significant difference between the population of *B. gladioli* KACC18962 in rice inoculated with single or mixed cultures.

Discussion

Since the first report of rice panicle blight and grain rot, the significance of rice diseases caused by *Burkholderia* spp. has continually increased and is expected to cause more severe losses with the impact of climate change (Schaad 2008). Most studies of plant diseases have been conducted using single-pathogen infections; however, under natural conditions, the host is often affected by several pathogens, some of which belong to the same group. *B. glumae*, *B. plantarii*, and *B. gladioli* share the same habitat and there have been reports of co-occurrence among them (Fory et al. 2014; Nandakumar et al. 2009). Therefore, studying rice diseases with reference to the interactions among these three species will contribute to understanding of the associated natural epidemiology. In this study, measurable antagonism among these three *Burkholderia* spp. was observed in several in vitro assays. These observed antagonistic interactions were verified in planta by performing rice stem and seedling coinfection assays and evaluating disease development and bacterial colonization of the internal rice tissues.

B. gladioli KACC18962 demonstrated a strong inhibitory effect on the other two *Burkholderia* spp. tested, both in disc diffusion and confirmatory cocultivation assays. The generation of GFP-expressing strains facilitated the study of interactions among the three species, particularly the inhibitory effect of *B. gladioli* KACC18962 on *B. glumae* BGR1 and *B. plantarii* KACC18965. The in vitro antibacterial activity of *B. gladioli* was previously reported in several studies. Riera-Ruiz et al. (2018) reported the in vitro inhibition of *B. glumae* by *B. gladioli*, and Elshafie et al. (2017) confirmed the in vitro inhibitory activity of *B. gladioli* against *Bacillus megaterium* and *E. coli*. The strains in the latter study were also shown to produce various hydrolytic enzymes and bioactive compounds. The production of bioactive metabolites may explain the biocontrol efficiency of *B. gladioli* reported against several plant pathogens (Altindag et al. 2006; Andolfi et al. 2008). Intriguingly, a novel antibacterial compound (gladiolin) was isolated and characterized from a strain of *B. gladioli* and was found to be effective

Table 2. Quantification of rice-pathogenic *Burkholderia* species from the stem and seedling assays by quantitative PCR

Treatment	From infected rice stems				Treatment	From infected seedlings			
	Bglu10 ^x		Bglu8			Bglu10		Bpln2	
	C _T ^y	10 ⁴ copies/μl	C _T	10 ⁴ copies/μl		C _T	10 ⁴ copies/μl	C _T	10 ⁴ copies/μl
Control ^z	0	0	27.17 ± 1.26	0.004 (0.51)	Control ^z	25.51 ± 0.92	0.064 (0.04)	25.93 ± 0.20	0.240 (0.14)
<i>B. gladioli</i>	20.84 ± 1.59	1.527 (0.64)	–	–	<i>B. gladioli</i>	12.67 ± 0.26	191.306 (0.16)	–	–
<i>B. glumae</i>	–	–	14.69 ± 0.25	54.489 (0.24)	<i>B. plantarii</i>	–	–	16.52 ± 0.40	100.416 (0.26)
<i>B. gladioli</i> + <i>B. glumae</i>	19.96 ± 0.06	1.821 (0.04)	21.61 ± 0.32	0.236 (0.19)**	<i>B. gladioli</i> + <i>B. plantarii</i>	12.51 ± 0.54	222.226 (0.36)	18.09 ± 0.95	41.497 (0.46)*

^x Bglu10, Bglu8, and Bpln2 quantitative PCR primer pairs specific for detection of *B. gladioli* KACC18962, *B. glumae* BGR1, and *B. plantarii* KACC18965, respectively.

^y Values are presented as the mean ± SD ($n = 3$) or the mean (coefficient of variation) ($n = 3$). Dashes indicate not tested. Asterisks denote the following: * $P < 0.05$ (significant difference compared with sole inoculation with *B. plantarii*) and ** $P < 0.01$ (significant difference compared with sole inoculation with *B. glumae*; $n = 3$). C_T = cycle threshold.

^z Sterilized distilled water was used as the control.

against multidrug-resistant *Mycobacterium tuberculosis* (Song et al. 2017). In this study, CFS from *B. gladioli* KACC18962 was effective against *B. glumae* BGR1 and *B. plantarii* KACC18965, which is compatible with the production of antibacterial compounds. Production of bioactive metabolites is not the only strategy by which bacteria can exert inhibitory activity against other microbes; gram-negative bacteria may use other strategies, such as type VI secretion systems, to translocate poisonous effectors to other microbes (Basler et al. 2013). This could explain the differences between the effects of CFS and cocultivation observed here.

The differences in optimal growth temperatures for the *Burkholderia* species used in this study may explain the fluctuations in the responses to cocultivation or treatment with CFS. Temperature and quorum sensing were previously reported to play a major role in the physiological functions of *B. glumae*; for example, at temperatures lower than 30°C, production of toxoflavin (a major virulence factor) was inhibited (Jang et al. 2014). The optimum temperature for *B. glumae* BGR1 growth in vitro is 37°C; thus, *B. glumae* showed more tolerance to the inhibitory activity of *B. gladioli* KACC18962 and was more active against *B. plantarii* KACC18965 at 37°C in this study. Moreover, the antibacterial activity of *B. gladioli* KACC18962 was more pronounced at 28°C, which is optimal for its in vitro growth.

The strong in vitro inhibitory activity of *B. gladioli* KACC18962 against *B. glumae* BGR1 and *B. plantarii* KACC18965 was confirmed in rice plants by performing stem and seedling assays, respectively. Coinfection with *B. gladioli* KACC18962 resulted in attenuation of the symptoms caused by *B. glumae* BGR1 and *B. plantarii* KACC18965 on rice stems and seedlings. The significant reduction in disease severity observed in this study was attributed to the inhibitory activity of *B. gladioli* against *B. glumae* and *B. plantarii*, which was verified using qPCR. These results are in accordance with those of previous studies on the influence of *B. gladioli* on other rice-pathogenic *Burkholderia* spp. Miyagawa (2000) reported that *B. gladioli* significantly suppressed the population of *B. plantarii* in germinating rice seeds. Moreover, in agreement with our results, Riera-Ruiz et al. (2018) reported dominance of *B. gladioli* rice colonization over *B. glumae*, in which recovery of *B. gladioli* from coinoculated seedlings was significantly higher than the recovery of *B. glumae*.

qPCR is one of the most widely used techniques in environmental microbiology for quantification of target genes in a particular sample. The qPCR developed in this study was based on primer pairs that were originally designed using a comparative genomics approach. This has been shown to be effective for designing species-specific primers for the detection of pathogenic microbes, particularly for differentiation between closely related species (Baek et al. 2018; Lee et al. 2018). Screening and melting curve analysis were used to select efficient primer pairs for qPCR of *Burkholderia* spp. from infected plant tissues, using a standard curve method for absolute quantification (Brankatschk et al. 2012). The qPCR was successfully applied to study the inhibitory activity of *B. gladioli* KACC18962 against *B. glumae* BGR1 and *B. plantarii* KACC18965 in tissues of inoculated rice stems and seedlings. The assays developed in this study will be useful for studying the epidemiology and behavior of these three rice-pathogenic *Burkholderia* spp. in rice plants and for accurately quantifying bacterial load.

In this study, we investigated the in vitro and in planta interactions among three rice pathogens: *B. glumae*, *B. plantarii*, and *B. gladioli*. Antagonism was observed among the three species, with *B. gladioli* showing strong inhibitory activity against *B. glumae* and *B. plantarii* in in vitro and in planta assays. Coinfection with *B. gladioli* resulted in suppression of *B. glumae* and *B. plantarii* populations in rice and thus significant attenuation of the associated disease development. Moreover, sole inoculation with *B. gladioli* on rice stems or seedlings results in weak disease. Taken together, these results suggest that *B. gladioli* represents a potential source of antibacterial compounds that could be utilized for management of *B. glumae* and *B. plantarii* infections in rice. Further work is required to identify the associated bioactive metabolites and any other antagonistic mechanisms of these three *Burkholderia* species.

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