The Virulence Function and Regulation of the Metalloprotease Gene *prtA* in the Plant-Pathogenic Bacterium *Burkholderia glumae*

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Bacterial panicle blight caused by *Burkholderia glumae* is a major bacterial disease of rice. Our preliminary RNA-seq study showed that a serine metalloprotease gene, *prtA*, is regulated in a similar manner to the genes for the biosynthesis and transport of toxoflavin, which is a known major virulence factor of *B. glumae*. *prtA* null mutants of the virulent strain *B. glumae* 336gr-1 did not show a detectable extracellular protease activity, indicating that *prtA* is the solely responsible gene for the extracellular protease activity detected from this bacterium. In addition, inoculation of rice panicles with the *prtA* mutants resulted in a significant reduction of disease severity compared with the wild-type parent strain, suggesting the requirement of *prtA* for the full virulence of *B. glumae*. A double mutant deficient in both serine metalloprotease and toxoflavin ($\Delta\text{toxA}/\text{prtA}^-$) exhibited a further numeric but not statistically significant decrease of disease development compared with the ΔtoxA strain. Both the *prtA*-driven extracellular protease activity and the toxoflavin production were dependent on both the *tofl/tofR* quorum-sensing and the global regulatory gene *qsmR*, indicating the important roles of the two global regulatory factors for the bacterial pathogenesis by this pathogen.

Keywords: bacterial panicle blight of rice, *Burkholderia glumae*, metalloprotease, quorum-sensing, *qsmR*, virulence factor

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Bacterial panicle blight (BPB) is one of the most severe bacterial diseases of rice (Ham et al. 2011). Since first reported in 1956 in Japan (Goto and Ohata 1956), this disease has been reported in many rice-producing areas worldwide (Cui et al. 2016; Ham et al. 2011). *Burkholderia glumae*, known as a seed-borne pathogen, is the major causal agent of BPB, which can survive both internally and externally of rice seeds (Nandakumar et al. 2009; Tsushima 1996). This pathogen also epiphytically grows on rice plants during the booting stage and multiplies on the surface of emerging panicles (Goto 1992).

Infection of rice plant by *B. glumae* is a multifactorial process requiring numerous virulence factors such as toxoflavin, lipase, type III secretion effectors, and flagella (Ham et al. 2011). Toxoflavin, a phytotoxin, is one of the most important virulence factors of *B. glumae*, and the genes for toxoflavin biosynthesis and transport are encoded by the *toxABCDE* and *toxFGHI* operons, respectively (Kim et al. 2004). Toxoflavin-deficient strains exhibit substantially lower levels of virulence compared with the wild-type parent but still retain the ability to cause disease in rice (Kim et al. 2004). Substantial reduction of virulence was also reported with the *B. glumae* strains deficient in lipase production (Devescovi et al. 2007) or flagella formation (Kim et al. 2007). Moreover, *B. glumae* is able to elicit hypersensitive responses in tobacco leaves in a type III secretion system (T3SS)-dependent manner (Kang et al. 2008). Type III effectors also contribute to the virulence of *B. glumae*, as indicated by the reduced virulence of a T3SS-defective mutant, ΔhrpB (Kang et al. 2008).

In *B. glumae*, major virulence factors are regulated by the *tofl/tofR* quorum-sensing (QS) system, which is mediated by the *luxI* and *luxR* homologs *tofl* and *tofR*, respectively (Chun et al. 2009; Kim et al. 2004, 2007). *tofl* encodes an acyl-homoserine lactone synthase responsible for synthesizing the QS signal compound, *N*-octanoyl homoserine lactone (C8-HSL), and *tofR* encodes a cognate receptor that C8-HSL binds to (Dunlap 1999; Kim et al. 2004). The *tofl/tofR* QS system regulates the expression of *toxR* and *toxJ*, which are additional regulatory elements required for the biosynthesis and transport of toxoflavin (Kim et al. 2004, 2007). The *tofl/tofR* QS system is also essential for flagellar biogenesis and lipase activity in *B. glumae* (Devescovi et al. 2007; Kim et al. 2007). Nickzad et al. (2015) reported that the *tofl/tofR* QS also regulates rhamnolipid production, which is required for swarming motility.

qsmR, which encodes an IclR-family transcriptional factor was first identified in *B. glumae* BGR1 as a QS ‘master regulator’ that controls flagellar biogenesis and flagellum-mediated motility (Kim et al. 2007). In *B. glumae* BGR1, *qsmR* exerts its

regulatory function through *flhD/flhC* and its expression is dependent on the *tofl/tofR* QS (Jang et al. 2014; Kim et al. 2007). Disruption of *qsmR* also caused substantial reduction of toxoflavin in liquid Luria Bertani (LB) medium but no difference was observed under the LB agar medium condition, indicating the regulatory role of *qsmR* in the toxoflavin production in a growth condition-dependent manner (Kim et al. 2007). Previous studies with *B. glumae* BGR1 also revealed that *qsmR* plays a pivotal role in the QS-dependent regulation of metabolic pathways to survive in stress conditions including the high cell density at the stationary phase (An et al. 2014; Goo et al. 2017). Most importantly, the near-avirulent phenotype of the *qsmR* null mutant observed in the same study indicates that *qsmR* is a key regulatory element governing the bacterial pathogenesis by *B. glumae* (Kim et al. 2007).

Our preliminary RNA-seq study with *B. glumae* 336gr-1 (a virulent strain in the United States) showed a serine metalloprotease gene being downregulated in a *B. glumae* mutant derivative deficient in the *tofl/tofR* QS system (Supplementary Table S1). The serine metalloprotease gene corresponds to

'*bglu_1g16590*' of the reference strain, BGR1, and was named *prtA*. In this study, we investigated the role of *prtA* in the extracellular protease activity and the virulence of *B. glumae* as well as its regulation by the *tofl/tofR* QS system and *qsmR*.

RESULTS

prtA encoding a serine metalloprotease was solely for the extracellular protease activity of *B. glumae* 336gr-1.

According to the genome sequence information of *B. glumae* BGR1 (Lim et al. 2009) and *B. glumae* 336gr-1 (Francis et al. 2013), *prtA* (*bglu_1g16590*) is located between the putative genes encoding glycogen synthase (*bglu_1g16580*) and the α/β hydrolase fold protein (*bglu_1g16600*) (Fig. 1A). *B. glumae* 336gr-1 exhibited a distinct extracellular protease activity on nutrient agar (NA) supplemented with 1% skim milk. The bacterial extracellular protease activity is indicated by the clear zone surrounding the bacterial colony as a result of protein digestion (Fig. 1B). To test if *prtA* contributes to the extracellular protease activity of *B. glumae* 336gr-1, null mutants of *prtA* were generated through insertional mutagenesis, using an

Fig. 1. The role of the *prtA* gene in the extracellular protease activity of *Burkholderia glumae* 336gr-1. **A**, A physical map of *prtA* that depicts its location, clones, and null mutants. The arrows represent the direction of the size of the corresponding genes, which are indicated by the gene ID numbers of the reference genome *B. glumae* BGR1 (Lim et al. 2009). **B**, Extracellular protease activities of 336gr-1 (wild type), a *prtA*⁻ mutant (LSUPB586-1), and LSUPB586-1 carrying pBB5prtA (a *prtA* clone) on a nutrient agar plate supplemented with 1% skim milk. Restriction sites used for the DNA constructs are *NotI* (N), *EcoRI* (R), and *XbaI* (X). Restriction sites of the cloning vectors are presented in parentheses.

internal fragment of *prtA* cloned in the suicide vector pKNOCK-Km (Fig. 1A). As shown in Figure 1B, LSUPB586-1, a representative *prtA*⁻ derivative of 336gr-1, did not exhibit any detectable extracellular protease activity on the NA supplemented with 1% skim milk. The lost extracellular protease activity in LSUPB586-1 was restored by a *prtA* clone, pBB5prtA, indicating that *prtA* is the solely responsible gene for the extracellular protease activity of *B. glumae* 336gr-1 (Fig. 1B).

The deduced protein sequence of PrtA contained a typical N-terminal signal sequence and its extracellular protease activity was dependent on the type II secretion system (T2SS).

Analysis of the deduced PrtA amino-acid sequence, using SignalP-4.1 (Petersen et al. 2011), revealed an N-terminal secretion signal peptide (Supplementary Fig. S1A). The cleavage site was predicted to be between the 27th (Ala) and the 28th (Gln) amino acid, with maximum C and Y scores of 0.801 and 0.869, respectively, at Q28 and maximum S score of 0.987 at L17. The mean S and D scores of the predicted signal peptide (the first 27 amino acids) were 0.949 and 0.907, respectively. As substrates of a T2SS need a signal peptide to pass through the inner membrane (Sandkvist, 2001), the PrtA metalloprotease is very likely to be secreted via a T2SS. According to the genome analysis using the web-based bioinformatics tool The Seed Viewer (Overbeek et al. 2005), *B. glumae* contains at least one gene cluster for a T2SS (Supplementary Fig. S2). To test the requirement of the T2SS for the extracellular activity of PrtA, the *gspD* and *gspE* genes that encode key components of the T2SS were disrupted individually through single homologous recombination on each gene (Supplementary Fig. S3). Both *gspD* and *gspE* mutants exhibited substantially less protease activity compared with the wild type, indicating that the extracellular activity of PrtA is dependent on secretion via the T2SS (Fig. 2A and B).

The extracellular protease activity of *B. glumae* 336gr-1 was dependent on the *tofl/tofR* QS system.

As major virulence factors of *B. glumae* (i.e., toxoflavin, lipase, flagella) are regulated by the *tofl/tofR* QS, we further investigated if the extracellular protease activity is under the regulation of *tofl/tofR* QS. In the genome of *B. glumae*, *tofl* and *tofR* are adjacent to each other with another regulatory gene

tofM between the two QS genes (Chen et al. 2012) (Fig. 3A). The four different deletion mutants, LSUPB145 ($\Delta tofI$), LSUPB169 ($\Delta tofR$), LSUPB286 ($\Delta tofM$), and LSUPB139 ($\Delta tofI$ - $\Delta tofR$) (Chen et al. 2012) (Fig. 3A), were tested for their extracellular protease activities along with the wild-type strain 336gr-1 (Fig. 3B and C). As shown in Figure 3B and C, the extracellular protease activity of *B. glumae* 336gr-1 was not significantly different from the $\Delta tofI$, $\Delta tofR$, or $\Delta tofM$ derivative. However, the extracellular protease activity was not detectable in the same conditions with LSUPB139, a derivative of 336gr-1 lacking the entire *tofl/tofM/tofR* cluster (Fig. 3B and C), and this protease-deficient phenotype of the mutant was restored by pBBtoflMR, a plasmid carrying *tofl/tofM/tofR* (Supplementary Fig. S4).

***prtA* was required for the full virulence of *B. glumae* 336gr-1.**

Virulence function of *prtA* for *B. glumae* in rice was examined with three independent *prtA*⁻ derivatives of 336gr-1, LSUPB586-1, LSUPB586-2, and LSUPB586-3, comparing them with wild-type parent 336gr-1 (Fig. 4A). All the *prtA*⁻ derivatives tested exhibited a significant reduction in disease severity (Fig. 4A). Further tests to verify the virulence function of *prtA* were conducted with additional strains, including LSUPB586-1(pBB5prtA) (a *prtA*⁻ strain complemented with the *prtA* clone pBB5prtA), LSUPB537 (a $\Delta toxA$ derivative of 336gr-1 defective in toxoflavin production), and LSUPB633 (a $\Delta toxA/prtA$ ⁻ derivative of 336gr-1 defective in both toxoflavin production and extracellular protease activity). As shown in Figure 4B, the *prtA*⁻ strain LSUPB586-1 was significantly less virulent than the wild-type parent 336gr-1, while LSUPB586-1(pBB5prtA) exhibited a level of virulence comparable to the wild type. This result indicates that the *prtA* clone pBB5prtA restored the impaired virulence of the *prtA* mutant, verifying the requirement of the *prtA* gene for the full virulence of *B. glumae* 336gr-1. The $\Delta toxA$ strain LSUPB537 was significantly less virulent than the *prtA*⁻ strain LSUPB586-1, indicating that toxoflavin has a greater impact on the virulence of *B. glumae* than the PrtA protease (Fig. 4B). Moreover, the double mutant strain LSUPB633 defective in both toxoflavin production and extracellular protease activity exhibited a further reduction in virulence compared with the $\Delta toxA$ strain LSUPB537, although the level of reduction was not statistically significant (Fig. 4B).

Fig. 2. Extracellular protease activities of *Burkholderia glumae* 336gr-1 and its *gspD* and *gspE* null mutant derivatives. **A**, Extracellular protease activities of wild type (WT, 336gr-1) and its *gspD*⁻ (LSUPB640) and *gspE*⁻ (LSUPB641) derivatives on a nutrient agar plate supplemented with 1% skim milk. **B**, Quantified protease activities of the cell-free culture filtrates from the same set of *B. glumae* strains grown in Luria Bertani broth. Protease activity was quantified following the method of Chessa et al. (2000). LSUPB586-1, a *prtA*⁻ strain, was used as a negative control.

***toxJ* and *toxR*, the regulatory genes required for toxoflavin production, were not involved in the extracellular protease activity of PrtA.**

In the previous study, by Kim et al. (2004), with *B. glumae* BGR1, *toxJ* and *toxR* were shown to activate the genes for toxoflavin biosynthesis and transport under the regulation of the *tofl/tofR* QS genes. To determine if *toxJ* and *toxR* are also involved in the extracellular protease activity of *B. glumae* 336gr-1, the $\Delta toxJ$ and $\Delta toxR$ derivatives of *B. glumae* 336gr-1 (named LSUPB592 and LSUPB590, respectively) were generated and their extracellular protease activities were examined. Both $\Delta toxJ$ and $\Delta toxR$ strains exhibited comparable levels of extracellular protease activities to the wild type regardless of solid or liquid medium condition, while the negative control (the QS-deficient mutant LSUPB139) did not express any observable extracellular protease activity (Fig. 5A and B). In

contrast, toxoflavin production was abolished in the $\Delta toxJ$ and $\Delta toxR$ strains like in the QS-deficient mutant (Fig. 5C and D), which was congruent with the previous study with *B. glumae* BGR1 (Kim et al. 2004). These results indicate that the extracellular protease activity of *B. glumae* 336gr-1 is not dependent on the regulatory genes for toxoflavin production, *toxJ* and *toxR*, while the *tofl/tofR* QS genes globally regulate both virulence functions.

***qsmR* was required for both extracellular protease activity and toxoflavin production.**

qsmR encoding an IclR-family protein is another regulatory element known to play a crucial role for the virulence of *B. glumae* BGR1 (Kim et al. 2007). In this study with *B. glumae* 336gr-1, the $\Delta qsmR$ derivative LSUPB574 completely lost its extracellular protease activity and toxoflavin production, like

Fig. 3. Extracellular protease activities of *Burkholderia glumae* 336gr-1 and its quorum-sensing mutant derivatives. **A**, A schematic description of the quorum-sensing mutants tested in this study. **B**, Extracellular protease activities of the wild type (WT) and the quorum-sensing mutant strains on nutrient agar plates supplemented with 1% skim milk. **C**, Quantified protease activities of the cell-free culture filtrates from the same set of *B. glumae* strains grown in Luria Bertani broth. Protease activity was quantified following the method of Chessa et al. (2000). The strain for each genotype is 336gr-1 (WT), LSUPB145 ($\Delta tofl$), LSUPB286 ($\Delta tofM$), LSUPB169 ($\Delta tofR$), and LSUPB139 ($\Delta tofl-tofR$). LSUPB586-1, a *prtA*⁻ strain, was used as a negative control, and pBB5prtA is a *prtA* clone that complements the *prtA* mutant.

the QS-deficient mutant LSUPB139 (Fig. 6A, B, and C). In contrast, LSUPB145 and LSUPB169 (the $\Delta tofI$ and $\Delta tofR$ derivatives of *B. glumae* 336gr-1, respectively) retained the wild-type level of extracellular protease activity in both NA agar and LB medium conditions (Fig. 6A and B), although their toxoflavin production was substantially reduced when they were grown in LB broth (Fig. 6C). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) tests revealed that the transcription of *prtA* was abolished in

LSUPB139 ($\Delta tofI$ - $\Delta tofR$) and LSUPB574 ($\Delta qsmR$) (Fig. 6D and E).

The expression of *qsmR* was not affected by *tofI/tofR* QS, while *qsmR* partially affected the expression of *tofR*, *toxJ*, and *toxR* but not the expression of *tofI*.

To determine the impact of *tofI/tofR* QS on the expression of *qsmR*, the transcriptional level of *qsmR* was accessed and compared between the wild type (336gr-1) and its $\Delta tofI$ - $\Delta tofR$

Fig. 4. The virulence function of *prtA* for *Burkholderia glumae* 336gr-1 in rice panicles. **A**, Disease progress curves of rice panicles inoculated with *B. glumae* 336gr-1 and its three independent *prtA*⁻ derivatives, LSUPB586-1 (*prtA*⁻1), LSUPB586-2 (*prtA*⁻2) and LSUPB586-3 (*prtA*⁻3). **B**, Disease progress curves of rice panicles inoculated with *B. glumae* 336gr-1 and its derivatives: LSUPB586-1 (*prtA*⁻), LSUPB586-1 carrying a *prtA* clone [*prtA*⁺ (pBB5prtA)], LSUPB537 ($\Delta toxA$), and LSUPB633 ($\Delta toxA$ /*prtA*⁻). Disease severity was determined based on a 0 to 9 scale: 0 = no symptom, 1 = 1 to 10% symptomatic area, 2 = 11 to 20% symptomatic area, 3 = 21 to 30% symptomatic area, 4 = 31 to 40% symptomatic area, 5 = 41 to 50% symptomatic area, 6 = 51 to 60% symptomatic area, 7 = 61 to 70% symptomatic area, 8 = 71 to 80% symptomatic area, and 9 = more than 80% symptomatic area. The susceptible rice variety Trenasse was inoculated to evaluate the virulence of each strain. Error bars indicate the standard deviations from 15 replications. Similar results were also obtained from an additional two independent experiments. The letters at the end of individual data curves indicate statistically significant differences among the data curves at $P < 0.05$, based on Tukey's posthoc test.

Fig. 5. The differential regulatory functions of *toxJ* and *toxR* in extracellular protease activity and toxoflavin production of *Burkholderia glumae* 336gr-1. **A**, Extracellular protease activities of the wild type (WT) and its $\Delta toxJ$, $\Delta toxR$, and $\Delta tofI$ - $\Delta tofR$ derivatives on a nutrient agar plate supplemented with 1% skim milk. **B**, Quantified protease activities of the cell-free culture filtrates from the same set of *B. glumae* strains grown in Luria Bertani broth. Protein activity was quantified following the method of Chessa et al. (2000). **C**, Toxoflavin-producing phenotypes of the WT and its $\Delta toxJ$, $\Delta toxR$, and $\Delta tofI$ - $\Delta tofR$ derivatives indicated by the yellow color of each cell culture. **D**, The toxoflavin-producing phenotypes of the same set of strains quantified based on absorbance at 393 nm (Kim et al. 2004). The strain for each genotype is 336gr-1 (WT), LSUPB590 ($\Delta toxR$), LSUPB592 ($\Delta toxJ$), and LSUPB139 ($\Delta tofI$ - $\Delta tofR$).

derivative (LSBPB139) with qRT-PCR. As shown in Figure 7A, there was no significant difference in the expression level of *qsmR* between the wild type and its $\Delta tofI$ -*tofR* derivative, indicating that *qsmR* expression is not affected by *tofI*/*tofR* QS in *B. glumae* 336gr-1. Likewise, *tofI* expression in the $\Delta qsmR$ genotype (LSUPB574) was not significantly different from that in the parent strain (336gr-1), indicating that *qsmR* does not have a strong impact on the expression of *tofI*, either (Fig. 7B).

However, expression levels of other regulatory genes (*tofR*, *toxJ*, and *toxR*) were significantly reduced in the $\Delta qsmR$ derivative, indicating that *qsmR*, if not completely, partially regulates those regulatory genes (Fig. 7B). Meanwhile, expression of *toxA* (a structural gene for toxoflavin biosynthesis) was completely abolished in both $\Delta qsmR$ and $\Delta tofI$ -*tofR* backgrounds, which was congruent with the toxoflavin production phenotypes of these mutant strains (Fig. 7A and B).

Fig. 6. The regulatory function of *qsmR* in extracellular protease activity and toxoflavin production of *Burkholderia glumae* 336gr-1. **A**, The extracellular protease activities of the wild type (WT) and its $\Delta qsmR$, $\Delta tofI$, $\Delta tofR$, $\Delta tofI$ -*tofR*, and *prtA*⁻ derivatives on a nutrient agar plate supplemented with 1% skim milk. **B**, Quantified protease activities of cell-free culture filtrates from the same set of *B. glumae* strains grown in Luria Bertani broth. Protein activity was quantified following the method of Chessa et al. (2000). **C**, Toxoflavin-producing phenotypes of the WT and its $\Delta qsmR$, $\Delta tofI$, $\Delta tofR$, and $\Delta tofI$ -*tofR* derivatives, quantified based on observance at 393 nm (Kim et al. 2004). The strain for each genotype is 336gr-1 (WT), LSUPB574 ($\Delta qsmR$), LSUPB145 ($\Delta tofI$), LSUPB169 ($\Delta tofR$), LSUPB139 ($\Delta tofI$ -*tofR*), and LSUPB586-1 (*prtA*⁻). **D**, Expression levels of *prtA* determined by qRT-PCR in 336gr-1 (WT), LSUPB145 ($\Delta tofI$), LSUPB169 ($\Delta tofR$), and LSUPB139 ($\Delta tofI$ -*tofR*). **E**, Expression levels of *prtA* determined by qRT-PCR in 336gr-1 (WT) and LSUPB574 ($\Delta qsmR$).

Fig. 7. Reciprocal influences between *qsmR* and *tofI*/*tofR* determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). **A**, The expression levels of *qsmR* in the wild type (WT) and its $\Delta tofI$ -*tofR* derivative. **B**, The expression levels of *tofI*, *tofR*, *toxJ*, and *toxR* in the WT and its $\Delta qsmR$ derivative. Expression of *toxA* was also determined as a control for both A and B. The strain for each genotype is 336gr-1 (WT), LSUPB139 ($\Delta tofI$ -*tofR*), and LSUPB574 ($\Delta qsmR$). Fold change of each gene in qRT-PCR was calculated by the $2^{-\Delta\Delta Ct}$ method, and expression was normalized using the reference genes *gyrA* and *16S*. Error bars represent standard deviation, and asterisks (*) indicate significant differences among data at $P < 0.05$, based on Tukey's studentized range test.

Genetic elements involved in the extracellular protease activity of *B. glumae* 336gr-1 were identified through screening of random mini-Tn5Km mutants.

Among approximately 4,000 random mutants screened on NA-1% skim milk plates, six mutants showed significantly altered extracellular protease activity through repeated tests (Table 1). Among the six mutants screened, four mutants exhibited significantly reduced extracellular protease activities compared with the wild-type parent 336gr-1, while two mutants did not show any detectable extracellular protease activity (Table 1). One of the four mutants showing reduced extracellular protease activities had a mini-Tn5Km insertion in a putative gene likely involved in signal transduction (336gr-1mr4) (Table 1). Two extracellular protease-deficient strains, 336gr-1mr1 and 336gr-1mr5, were found to be disrupted in putative genes encoding a transcriptional regulator LysR family and an arginyl-tRNA-protein transferase, which are likely to be involved in transcriptional regulation process and posttranslational regulation, respectively (Table 1).

DISCUSSION

In this study, we found that extracellular protease activity, encoded by *prtA*, is another critical virulence factor of *B. glumae*. According to the assay system used in this study, *prtA* appears to be solely responsible for the extracellular protease activity of *B. glumae* 336gr-1, as determined by the diminished enzymatic activity of a *prtA* null mutant on NA-1% skim milk plates. Analysis of the putative sequence of PrtA predicted the first 27 N-terminus amino acids as an N-terminal signal peptide for *sec*-dependent protein secretion, implying that this extracellular protease is secreted via a T2SS (Korotkov et al. 2012). Substantial reduction of the extracellular protease activity in the two T2SS-deficient mutant backgrounds (*gspD*[−] and *gspE*[−]) proved this notion, although residual enzymatic activities were detected with those secretion mutants (Fig. 2). As the T2SS exerts its function on the proteins exported from the cytoplasm to the periplasm via the *sec* or *tat* machinery, the observed residual activities are likely from the leakage of the PrtA proteases arrested in the periplasm of the *gspD* or *gspE* mutant. Blast search revealed the homologs of the PrtA protease among *Burkholderia* spp., including *B. gladioli* (another causal agent of BPB in rice) and other plant and animal pathogenic species (Supplementary Fig. S5). Interestingly, another serine metalloprotease (MrpA) is also present in *B. glumae*. However, this protease does not contain a signal sequence, suggesting that its enzymatic function is limited to the cytoplasm but not related to the extracellular protease activity of *B. glumae*.

A previous proteomic study by Goo et al. (2010) presented the idea that 34 extracellular proteins including the serine metalloprotease (*prtA*) were produced under the regulation of the *tofl/tofR* QS and were secreted via the T2SS in *B. glumae* BGR1. In that study, type II secretion-defective mutants (*gspC*[−], *gspE*[−], and *gspJ*[−]) exhibited substantial reductions in disease severity, indicating the importance of the T2SS-dependent extracellular proteins in the virulence of *B. glumae* BGR1. Our present study supports this and strongly suggests that *prtA* is a major virulence factor among the T2SS-dependent extracellular proteins of *B. glumae*.

Extracellular proteases play critical roles in bacterial pathogenesis (Frees et al. 2013; Lantz 1997), and several plant pathogens are known to rely on extracellular proteases for their pathogenic behaviors. A protease-deficient mutant of *Xanthomonas campestris* pv. *campestris* showed reduced ability to cause black rot systems on turnip leaves (Dow et al. 1990). In *Xanthomonas oryzae* pv. *oryzicola*, *ecpA* encoding an extracellular protease functions as a virulence factor in rice (Zou

et al. 2012). Virulence functions of extracellular proteases were also reported in the studies of soft rot-causing plant-pathogenic bacteria (Marits et al. 1999; Shevchik et al. 1998). Interestingly, a recently characterized extracellular protease gene in *Xylella fastidiosa*, *prtA*, was found to act as an antivirulence factor, regulating cell growth, biofilm formation, and pathogenicity (Gouran et al. 2016), suggesting the complex functions of extracellular protease in bacterial pathogenesis. To the best of our knowledge, the present study demonstrates the virulence function of an extracellular protease in plant-pathogenic *Burkholderia* species for the first time, although the zinc metalloproteases ZmpA and ZmpB were previously reported as potential virulence factors of the animal pathogenic strains of *B. cepacia* and *B. cenocepacia* (Corbett et al. 2003; Kooi et al. 2006).

In *B. glumae*, major virulence factors are regulated by the QS system mediated by *tofl* and *tofR* (Chen et al. 2012; Chun et al. 2009; Kim et al. 2007). Results of this study indicated that the extracellular protease activity was also dependent upon the *tofl/tofR* QS. The deletion of the entire *tofl/tofR* QS gene cluster, including *tofl*, *tofM*, and *tofR*, abolished the extracellular protease activity in the assay system used in this study. However, the single-gene mutants for the individual *tofl*, *tofM*, and *tofR* genes (LSUPB169, LSUPB 286, and LSUPB145, respectively) retained the enzymatic activity without any significant difference from wild type (Figs. 3 and 6). This observation is reminiscent of our previous data on toxoflavin production, in which toxoflavin production was completely inhibited by the deletion of the entire *tofl/tofR* QS gene cluster, while single-gene deletion mutants LSUPB145 ($\Delta tofI$) and LSUPB169 ($\Delta tofR$) still produced comparable amounts of toxoflavin on LB agar plates (Chen et al. 2012). In that study, deletion of *tofM* in the $\Delta tofI$ or $\Delta tofR$ background caused a substantial reduction of toxoflavin production on LB agar, suggesting that *tofM* plays a role as a pivotal ‘modulator’ in the *tofR*- and *tofl*-independent production of toxoflavin production by LSUPB145($\Delta tofI$) and LSUPB169 ($\Delta tofR$), respectively. The results of extracellular protease activity and toxoflavin production in our present and previous studies suggest the presence of unknown signaling and regulatory pathways in *B. glumae* 336gr-1 that regulate these virulence functions in conjunction with the *tofl/tofR* QS system including *tofM* (Fig. 8). The extracellular protease activity was not affected by *toxJ* and *toxR*, which are regulatory genes essential for toxoflavin production (Fig. 5), indicating that specific regulatory pathway for each virulence function branches out following the *tofl/tofR* QS. Collectively, it can be speculated that, in *B. glumae* 336gr-1, the two virulence functions (extracellular protease activity and toxoflavin production) share a common signaling and regulatory pathway involving the *tofl* and *tofR* genes, but decoupling also occurs conditionally in the

Table 1. Mini-Tn5Km mutants of *Burkholderia glumae* 336gr-1 showing altered phenotypes in extracellular protease activity

Strain name	Locus tag ^a	Phenotype ^b	Gene disrupted
336gr-1mr1	bglu_1g34140	NP	Transcriptional regulator, LysR family
336gr-1mr2-1	bglu_1g31090	–	Excinuclease ABC subunit A
336gr-1mr2-2	–	–	–
336gr-1mr3	bglu_1g02410	–	Phenylacetate-CoA oxygenase subunit PaaA
336gr-1mr4	bglu_1g10190	–	Diguanylate cyclase/phosphodiesterase
336gr-1mr5	bglu_1g19530	NP	Arginyl-tRNA-protein transferase

^a Corresponding gene ID numbers of the reference genome BGR1.

^b No extracellular protease activity (NP) and reduced extracellular protease activity (–) compared to wild-type parent 336gr-1.

absence of *tofI* or *tofR* and in the downstream pathways specific to each virulence function. The regulatory pathway and its regulatory elements specific for the extracellular protease activity are currently being characterized, following the screening of random mutants with altered extracellular protease activity.

In previous seminal studies on *B. glumae* BGR1, *qsmR* was characterized to be dependent on the *tofI/tofR* QS for its transcription and to have a pleiotropic regulatory function in flagellar biogenesis for motility and virulence, as well as in metabolic modulation for survival in stress conditions (An et al. 2014; Goo et al. 2017; Jang et al. 2014; Kim et al. 2007). An RNA-seq study of *B. glumae* BGR1, performed by Kim et al. (2013), also supports those studies, in which *tofI* showed more a important role in motility than *qsmR* did. In this study, however, transcription of *qsmR* appeared to be independent of the *tofI/tofR* QS in *B. glumae* 336gr-1 (Fig. 7A), indicating the presence of variation between the two *B. glumae* strains in the regulatory system. Another interesting fact found in this study was that *qsmR* functions as an essential regulatory factor for toxoflavin

production in *B. glumae* 336gr-1. In *B. glumae* BGR1, though disruption of *qsmR* caused a substantial reduction of toxoflavin production in LB broth, the *qsmR* mutant produced comparable amounts of toxoflavin to the wild-type parent in LB agar medium conditions (Kim et al. 2007). In *B. glumae* 336gr-1, however, toxoflavin production was almost abolished in the $\Delta qsmR$ background under both LB broth and LB agar medium conditions, indicating that both *qsmR* and the *tofI/tofR* QS are essential for this virulence function (Fig. 6C). Given the observation that *qsmR* only partially regulates *toxJ* and *toxR* (Fig. 7B), *qsmR* may also exert its regulatory function through an unknown regulatory pathway required for toxoflavin production. *qsmR* is also essential for the extracellular protease activity of *B. glumae* 336gr-1, indicating its global regulatory role for multiple virulence functions like the *tofI/tofR* QS.

According to a schematic model based on results from this study, both *tofI/tofR* QS including *tofM* and *qsmR* are required for toxoflavin production and extracellular protease activity

Fig. 8. A schematic model of the signaling/regulatory network for the virulence functions of *Burkholderia glumae* 336gr-1. The regulatory pathway through the *tofI/tofR* QS is indicated with blue arrows, while that through *qsmR* is indicated by red arrows. Partial influence of *qsmR* on the expression of *tofR*, *toxJ*, and *toxR* is indicated with hatched red arrows. According to the current model, both actions of the *tofI/tofR* QS and *qsmR* are required for the two virulence functions, toxoflavin production, and the PrtA protease activity. TofM is considered a pivotal modulator for the *tofI/tofR* QS function. C8-HSL = octanoyl-homoserine lactone (produced by *tofI*).

(Fig. 8). In the absence of *tofl* (or C8-HSL), one or more unknown signals may replace the function of C8-HSL, leading to the production of toxoflavin and the PrtA extracellular protease (Fig. 8). Likewise, in the absence of *tofR*, one or more unknown receptors of C8-HSL may replace the function of TofR to express virulence functions (Fig. 8). However, in the *tofl/tofR* QS-deficient or $\Delta qsmR$ background, *qsmR* or *tofl/tofR* QS alone, respectively, cannot make the expression of the virulence functions (Fig. 8). The unknown signaling molecules and the alternative QS signal receptor inferred from the model need to be identified through further comprehensive genetic and biochemical studies to elucidate the molecular mechanism underlying the expression of virulence functions in the absence of *tofl* or *tofR*.

The apparent difference between two strains of *B. glumae* (336gr1 versus BGR1) in the regulatory system involving the *tofl/tofR* QS and *qsmR* implies that a certain degree of variation exists in the signaling and regulatory systems among different strains of *B. glumae*. We previously presented the diverse phenotypic variations related to virulence and pigmentation among strains of *B. glumae* (Karki et al. 2012). Considering that *B. glumae* 336gr-1 and *B. glumae* BGR1 belong in the same clade based on genomic structure and phenotypes (Karki et al. 2012), it is very probable that the diversity of the regulatory network for virulence functions within the entire *B. glumae* species is even more profound than that observed in this study between the two strains. In fact, we recently found that another virulent United States strain of *B. glumae* produces toxoflavin even in the absence of the *tofl/tofR* QS (T. Lelis and J. H. Ham unpublished).

In addition, candidate genetic elements involved in the extracellular protease activity of *B. glumae* were newly identified in this study through screening of random mini-Tn5Km mutants. All the mutants characterized showed reduced or non-protease activities, suggesting that the mutated genes exert positive functions for the extracellular protease activity. At least some of the mutated genes identified in this study encode protein products known to function in signal transduction (e.g., diguanylate cyclase/phosphodiesterase), posttranslational regulation (e.g., arginyl-tRNA-protein transferase), and transcriptional regulation (e.g., LysR family transcriptional regulator), implying the high fidelity of this primary mutant screening process. Two other LysR family transcriptional regulators, *toxR* (Kim et al. 2004) and *ntpR* (Melanson et al. 2017), were previously characterized in *B. glumae*. *toxR* regulates toxoflavin biosynthesis (Kim et al. 2004) (Fig. 5) but not the extracellular protease activity (Fig. 5). *ntpR*, on the other hand, is a negative regulator for toxoflavin production (Melanson et al. 2017), but its function on extracellular protease has not yet been determined. Nevertheless, further genetic studies are required to verify the regulatory function of each candidate gene identified in this study.

Conclusively, this study demonstrates i) the extracellular metalloprotease gene *prtA* is a new virulence factor of *B. glumae*, ii) the regulation pattern of extracellular protease activity is similar to that of the major virulence factor toxoflavin in the United States strain *B. glumae* 336gr-1, iii) *qsmR* functions as an essential regulatory gene for both extracellular protease activity and toxoflavin production in *B. glumae* 336gr-1, and iv) the global regulatory network involving the *tofl/tofR* QS and *qsmR* varies significantly between *B. glumae* 336gr-1 and *B. glumae* BGR1. This study also strongly suggests that high levels of variation exist among different strains of *B. glumae* in the signaling and regulatory network for bacterial pathogenesis, which should be seriously considered when we attempt to design target-oriented management strategies for this pathogen based on its biological properties.

MATERIALS AND METHODS

Strains and culture conditions.

Bacterial strains and plasmids used in this study are listed in Supplementary Table S2. Bacterial strains were routinely grown in LB or LB agar medium, which was supplemented with antibiotics, as needed, at the following concentrations (per millimeter): 100 µg of ampicillin, 50 µg of kanamycin, 20 µg of gentamycin, and 100 µg of nitrofurantoin. DNA cloning and amplification were conducted following established methods (Sambrook and Russel 2001). PCR products were purified using the QuickClean 5M PCR purification kit (GenScript, Piscataway, NJ, U.S.A.). A NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.) was used to assess the quality and quantity of DNA samples. DNA sequencing of PCR products and DNA clones was performed by MacroGen USA (Rockville, MD, U.S.A.).

Generation of *prtA*, *gspD*, and *gspE* mutants and the *prtA* clone for complementation.

To generate a *prtA*::pKNOCK-Km mutant, a 403-bp internal fragment of *prtA* was amplified using MproTF1 and MprotR1 (Supplementary Table S3) and was ligated into the PCR cloning vector, psC-A-amp/kan. Using the *EcoRI* sites in the vector, the fragment was mobilized into the suicide vector pKNOCK-Km (Alexeyev 1999), generating pKnockprtA in *Escherichia coli* S17-1λpir (Fig. 1A). pKnockprtA was then conjugated into *B. glumae* via triparental mating, using the helper strain *E. coli* HB101 (pRK2013::Tn7). Colonies were selected based on antibiotic resistance to nitrofurantoin (for *B. glumae*) and kanamycin (for pKNOCK-Km inserted in *prtA*) on LB agar plates.

To complement the *prtA*::pKNOCK-Km mutant, 1,193-bp containing the promoter region and a 5' portion of *prtA* and a 1,497-bp containing the remaining part of *prtA* were amplified using the MetprAupF1/MetprAupR1 and MetprAdownF1/MetprAdownR1 primer pairs, respectively (Fig. 1A). The upstream and downstream PCR products were ligated separately into the PCR cloning vector psC-A-amp/kan, generating pTopometallUP and pTopometallDown, respectively. pTopometallDown was digested with *NotI* and *XbaI* and was subcloned into pKNOCK-Km suicide vector (Alexeyev 1999), generating pKkmMetallDown. pTopometallUP was digested with *NotI* and was cloned into pKkmMetallDown, generating pKkmprtA. pKkmprtA was digested with *EcoRI* and was cloned into broad-host range vector pBBR1MCS-5 (Kovach et al. 1994), generating pBB5prtA (Fig. 1A). pBB5prtA was transformed into *E. coli* S17-1λpir-competent cells through electroporation and was then conjugated into *B. glumae* via triparental mating, using the *E. coli* HB101 (pRK2013::Tn7) helper strain. Colonies were selected based on resistance to nitrofurantoin and gentamycin on LB agar plates.

To generate a *gspD*::pKNOCK-Km construct, a 541-bp internal fragment of *gspD* was amplified using TTssGspDF2 and TTssGspDR2. The resultant PCR product was then ligated to the PCR cloning vector psC-A-amp/kan. Using the *EcoRI* sites in the cloning vector, the cloned fragment was transferred to the suicide vector pKNOCK-Km (Alexeyev 1999), generating pKkmgspD in *E. coli* S17-1λpir. pKkmgspD was then conjugated into *B. glumae* via triparental mating using the helper strain *E. coli* HB101 (pRK2013::Tn7). Colonies were selected based on the antibiotic resistance to nitrofurantoin (for *B. glumae*) and kanamycin (for pKNOCK-Km carrying the internal fragment of *gspD*) on LB agar plates. To generate a *gspE*::pKNOCK-Km construct, a 468-bp internal fragment of *gspE* was amplified using TTssGspEF1 and TTssGspER1, followed by cloning into the PCR cloning vector psC-A-amp/kan. Using the *EcoRI* sites in the vector, the cloned

fragment was transferred to the suicide vector pKNOCK-Km (Alexeyev 1999), generating pKkmgspE in *E. coli* S17-1 λ pir. *gspE* was mutated in a same way as *gspD*, using the DNA construct pKkmgspE.

Generation of the Δ *toxA*, Δ *toxJ*, and Δ *toxR* derivatives of 336gr-1.

To generate LSUPB537, a Δ *toxA* derivative of 336gr-1, a 502-bp upstream flanking region, and a 481-bp downstream flanking region of *toxA* were amplified using primer sets ToxA UpF/ToxA UpR and ToxA DWNF/ToxA DWNR, respectively. The upstream and downstream PCR products were ligated separately into PCR cloning vector pSC-A-amp/kan. pSCtoxAup, the plasmid containing the upstream flanking sequence of *toxA*, was cleaved using *Bam*HI and *Spe*I, and was then subcloned into the suicide vector pKKSacB (pKKSacB-toxAup). pSCtoxAown, the downstream flanking sequence of *toxA*, was cleaved using *Spe*I and *Xba*I and was then cloned into pKKSacBtoxAup, generating pKKSacBtoxA. pKKSacBtoxA was transformed into *E. coli* S17-1 λ pir competent cells through electroporation and was then conjugated into *B. glumae* via triparental mating, using *E. coli* HB101 (pRK2013::Tn7) helper strain. Bacterial cells having single homologous recombination were selected based on antibiotic resistance to nitrofurantoin and kanamycin on LB agar plates. Subsequently, the selected colonies were grown overnight at 30°C in LB broth without any antibiotics and were spread on LB agar plates containing 30% sucrose to select mutants with secondary homologous recombination. Sucrose-resistant colonies were tested for sensitivity to kanamycin, and deletion of *toxA* was confirmed by PCR using the primer set ToxAcompF/ToxAcompR.

To generate LSUPB592, a Δ *toxJ* derivative of 336gr-1, a 415-bp upstream flanking region and a 540-bp downstream flanking region of *toxJ* were amplified using primer sets ToxJupFP/ToxJupRP and ToxJDwnFP/ToxJDwnRP, respectively. Both upstream and downstream PCR fragments were ligated separately into the PCR cloning vector pSC-A-amp/kan. pTopotoxJup carrying the upstream sequence of *toxJ* was cleaved using the *Xba*I and *Spe*I restriction sites, and the upstream sequence of *toxJ* was then subcloned into suicide vector pKKSacB to generate pKKSacBtoxJup. pTopotoxJDwn carrying the downstream sequence of *toxJ* was cleaved using *Spe*I and *Bam*HI, and the downstream sequence of *toxJ* was then ligated to pKKSacBtoxJup, generating pKKSacB Δ toxJ. pKKSacB Δ toxJ was transformed into *E. coli* S17-1 λ pir competent cells through electroporation and was then conjugated into *B. glumae* via triparental mating, using the helper strain *E. coli* HB101(pRK2013::Tn7). Selection of Δ *toxJ* derivatives was achieved through the procedure previously established (Chen et al. 2012). Deletion of *toxJ* was confirmed by PCR using the primer set ToxJcompF/ToxJcompR.

The *toxR* gene sequence is present upstream of the *tox* operon containing five putative toxoflavin biosynthetic genes, *toxA*, *toxB*, *toxC*, *toxD*, and *toxE*. To generate LSUPB590, a Δ *toxR* derivative of 336gr-1, the plasmid carrying a *toxA* upstream sequence, pKKSacBtoxAup, was used as the intermediate DNA construct for a *toxR* downstream sequence. For cloning an upstream region of *toxR*, a 408-bp upstream sequence of *toxR* was amplified using the primer set ToxRUpF/ToxRUpR. This PCR product was initially cloned in pSC-A-amp/kan, generating pTopotoxRup, and was subsequently ligated to pKKSacBtoxAup, using the *Bam*HI restriction sites. The resultant DNA construct pKKSacB Δ toxR was used for generating Δ *toxR* derivatives through the same procedure described above for deletion of *toxJ*. Deletion of *toxR* was confirmed by PCR using the primer set ToxRcompF/ToxRcompR.

Generation of the Δ *qsmR* derivative of 336gr-1.

A schematic illustration for *qsmR* deletion is presented in Supplementary Figure S6. The markerless *qsmR* deletion mutant LSUPB574 was generated through double-crossover homologous recombination in the flanking regions of the *qsmR* gene. A 458-bp upstream region and a 469-bp downstream region were amplified with the primer sets qsmrBamHIUL/qsmrSpeIUR and qsmrSpeIDL/qsmrXbaIIDR1. Resultant PCR products of the upstream and downstream regions were then cloned to pSC-A-amp/kan to generate pSCqsmRU and pSCqsmRD, respectively. The upstream region cloned in pSCqsmRU was further cloned to pKKSacB, using the *Bam*HI and *Spe*I restriction sites to generate pKKqsmRU. Finally, the *qsmR* downstream region present in pSCqsmRD was cloned to pKKqsmRU, using the *Spe*I and *Xba*I restriction sites to obtain pKKSacB Δ qsmR. The DNA construct for deletion of *qsmR*, pKKSacB Δ qsmR, was primarily transformed into *E. coli* S17-1 λ pir through electroporation and was then introduced into *B. glumae*, via triparental mating, using the helper strain *E. coli* HB101(pRK2013::Tn7). Selection of Δ *toxJ* derivatives was achieved through the procedure previously established (Chen et al. 2012). Deletion of *qsmR* was confirmed by PCR using the primer set qsmrDLcheckF/qsmrDLcheckR.

Extracellular protease assay.

Extracellular protease activity was initially assessed on NA plates amended with 1% skim milk, following the method of Huber et al. (2001) with some modifications. Briefly, 1 ml of an overnight culture of *B. glumae* incubated at 37°C was washed twice with fresh LB broth, using a microcentrifuge, and the final bacterial suspension was adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0. Five microliters of the bacterial suspension were spotted on an NA plate amended with 1% skim milk, followed by incubation at 37°C for 48 h. Extracellular protease activity was determined based on the halo zone formed around each bacterial colony.

A quantitative assay of extracellular protease activity was conducted using azocasein (Sigma, Saint-Louis) as the proteolytic substrate, following the method of Chessa et al. (2000). Briefly, 100 μ l of the cell-free supernatant from an overnight bacterial culture grown in LB broth was combined with 400 μ l of the protease assay buffer (composed of 100 μ l of 30 mg of azocasein per milliliter and 300 μ l of 20 mM Tris/1 mM CaCl₂, pH 8). The reaction solutions were incubated for 10 min at 20°C, followed by the addition of the stopping solution (500 μ l of 100 mg of trichloroacetic acid per milliliter). Following the subsequent centrifugation at 13,000 $\times$ g for 2 min, the absorbance of each supernatant was measured at 366 nm, using a spectrophotometer (Biomate 3, Thermo Electron Corp.). Fresh medium without bacterial cells was used for the blank control.

Toxoflavin quantification.

Toxoflavin production was measured following the previously established method (Kim et al. 2004) with minor modifications. Briefly, bacterial strains freshly retrieved from glycerol stocks stored at -80°C were grown in LB broth, for 24 h at 37°C, in a shaking incubator rotating at 200 rpm. Each bacterial culture was then centrifuged to extract the toxoflavin present in the supernatant. For toxoflavin extraction, 1 ml of the supernatant was mixed with 1 ml of chloroform through vortexing for 15 s. Each sample was centrifuged at 12,000 $\times$ g for 10 min to separate the chloroform phase. The chloroform phase was transferred to a new microtube and was placed in a fume hood overnight to evaporate. After evaporation, the remaining materials in the microtube including toxoflavin were resuspended in 1 ml of 80% methanol. Absorbance was measured at

393 nm (OD₃₉₃), using the BioMate 3 spectrophotometer (Thermo Electron Corp.). Fresh medium without bacterial cells instead of a bacterial culture was used for the blank control.

Virulence assay.

Virulence assay was conducted with a highly susceptible rice variety, Trenasse. Overnight cultures of *B. glumae* strains on LB agar plates were suspended in sterile tap water to a final concentration of approximately 5×10^7 CFU/ml (OD₆₀₀ = 0.1). Rice plants were inoculated twice in a 2-day interval with a hand sprayer when panicles were emerging. Disease progress was assessed five times during a 14-day period after inoculation. Statistical analysis for the virulence data sets was conducted with Tukey's post hoc tests using SAS version 9.3.

RNA extraction, cDNA synthesis, and qPCR.

B. glumae strains were cultured overnight in 10 ml of LB broth at 37°C in a shaking incubator rotating at 200 rpm. A 1-ml aliquot was washed twice with fresh LB broth and was resuspended in equal volumes of LB broth. Ten microliters of the resuspension were inoculated in 10 ml of LB broth and were incubated at 37°C until bacterial culture reaches OD₆₀₀ = 1.0. One milliliter of bacterial suspension was pelleted by centrifugation, was frozen in liquid nitrogen, and was resuspended in 1 ml of TRIzol reagent (Ambion Life Technologies, Grand Island, NY, U.S.A.). RNA extraction and DNase treatment were performed using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, U.S.A.), following manufacturer instructions. cDNA was prepared using iScript gDNA Clear cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), following manufacturer instructions. qPCR was performed using SsoAdvance Universal SYBR- Green supermix (Bio-Rad Laboratories, Inc.) following manufacturer instructions. Reactions were conducted in a Bio-Rad CFX Connect thermal cycler (Bio-Rad Laboratories, Inc.). The qPCR reaction program consisted of an initial denaturation step at 95°C for 60 s, followed by 39 cycles of 95°C for 15 s and 52°C for 20 s. A melt curve analysis of temperatures from 65°C to 95°C at 0.5°C increments was performed for each run. Expression values were normalized using two housekeeping genes, *gyrA* and *16S*. Two-tailed *t* test was performed for the statistical analyses of the qRT-PCR data using SAS version 9.3.

Sample preparation for RNA-seq.

A 1-ml overnight culture of *B. glumae* 336gr-1 and LSUPB139 ($\Delta tofI-R$) (Chen et al. 2012) was washed twice and was resuspended in the same volume of LB broth, and 15 μ l of the suspension was inoculated in 15 ml of LB and was incubated at 37°C until the early stationary phase (OD₆₀₀ = 1.0). Each bacterial culture (1 ml) was then pelleted and placed in liquid nitrogen for 5 to 10 s. The frozen pellet was resuspended in an equal volume of TRIzol reagent (Invitrogen) for RNA extraction. The extracted total RNA samples were then treated with DNA-free DNase treatment and removal reagents (Life Technologies) for the removal of residual DNA, following manufacturer instructions. RNA samples were then treated with a MEGAclear kit (Life Technologies) to eliminate the divalent cations left in the sample solution as well as the short oligonucleotides and protein molecules. RNA samples were then treated with a MICROBExpress bacterial mRNA enrichment kit (Life Technologies) for the removal of rRNA. The RNA samples were further processed with RNase III (Life Technologies) to reduce the fragmentation variability and with a RiboMinus Concentration Module (Life Technologies) to further eliminate rRNA. Library construction for RNA sequencing was performed using an RNA-Seq library construction kit (Life Technologies), following the manufacturer's instruction.

High-throughput DNA sequencing and sequence analysis.

High-throughput sequencing was conducted in the Virginia Bioinformatics Institute Core Lab, using the Illumina GAIIX platform for single-end sequencing for 50 cycles; 1.6 GB and 953.9 MB of sequence reads were generated from 336gr-1 and LSUPB139, respectively. To ensure high sequence quality, adaptors and low-quality sequences were trimmed using Trimmomatic (Bolger et al. 2014). The remaining sequences were mapped to the *B. glumae* BGR1 reference genome using Bowtie2 (Langmead and Salzberg 2012). Mapped reads were counted using HTSeq (Anders et al. 2015), and differential expression analyses were performed with edgeR (Robinson et al. 2010). Genes with a fold change in expression of ≥ 1.5 and a false discovery rate of ≤ 0.05 were considered to be differentially expressed.

Random mutagenesis of *B. glumae* using mini-Tn5Km.

Random mini-Tn5Km mutants of *B. glumae* were generated following a previously established method (Chen et al. 2012), through mixing a 3:1 ratio (vol/vol) of *E. coli* S17-1 λ pir (pUT::mini-Tn5Km) (de Lorenzo et al. 1990) and *B. glumae* 336gr-1. Mutants were screened based on altered phenotypes compared with the parent in the halo zone formation on NA agar supplemented with 1% skim milk. The locations of mini-Tn5Km insertion in the *B. glumae* genome were determined with a previously established method (Melanson et al. 2017), which was modified from the method described by Kwon and Ricke (2000).

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