tepR encoding a bacterial enhancer-binding protein orchestrates the virulence and interspecies competition of *Burkholderia glumae* through *qsmR* and a type VI secretion system

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Abstract
The pathogenesis of the rice pathogenic bacterium *Burkholderia glumae* is under the tight regulation of the *tofI/tofR* quorum-sensing (QS) system. *tepR*, encoding a group I bacterial enhancer-binding protein, negatively regulates the production of toxoflavin, the phytotoxin acting as a major virulence factor in *B. glumae*. In this study, through a transcriptomic analysis, we identified the genes that were modulated by *tepR* and/or the *tofI/tofR* QS system. More than half of the differentially expressed genes, including the genes for the biosynthesis and transport of toxoflavin, were significantly more highly expressed in the Δ*tepR* mutant but less expressed in the Δ*tofI-tofR* (*tofI/tofR* QS-defective) mutant. In consonance with the transcriptome data, other virulence-related functions of *B. glumae*, extracellular protease activity and flagellum-dependent motility, were also negatively regulated by *tepR*, and this negative regulatory function of *tepR* was dependent on the IclR-type transcriptional regulator gene *qsmR*. Likewise, the Δ*tepR* mutant exhibited a higher level of heat tolerance in congruence with the higher transcription levels of heat shock protein genes in the mutant. Interestingly, *tepR* also exhibited its positive regulatory function on a previously uncharacterized type VI secretion system (denoted as BgT6SS-1). The survival of the both Δ*tepR* and Δ*tssD* (BgT6SS-1-defective) mutants was significantly compromised compared to the wild-type parent strain 336gr-1 in the presence of the natural rice-inhabiting bacterium, *Pantoea* sp. RSPAM1. Taken together, this study revealed pivotal regulatory roles of *tepR* in orchestrating multiple biological functions of *B. glumae*, including pathogenesis, heat tolerance, and bacterial interspecies competition.

KEYWORDS
bacterial competition, bacterial panicle blight, *burkholderia glumae*, *qsmR*, quorum-sensing, *tepR*, type VI secretion system
INTRODUCTION

*Burkholderia glumae*, the major causal agent of bacterial panicle blight of rice, causes significant economic damage in many rice-growing areas across the world (Ham et al., 2011; Zhou, 2014). *B. glumae* has also been reported to cause wilting diseases in many other field crops, including tomato, hot pepper, potato, eggplant, sesame, and sunflower (Jeong et al., 2003). *B. glumae* is generally considered as a seedborne pathogen that colonizes rice seedlings and survives in host plants throughout the growing season, but it is also thought to infect hosts through the stomata or wounds and proliferate in the intercellular spaces of parenchyma (Hikichi, 1993; Ham et al., 2011).

*B. glumae* uses multiple virulence factors to facilitate its infection in rice plants (Kim et al., 2004, 2007; Suzuki et al., 2004; Devescovi et al., 2007; Lelis et al., 2019). The most important virulence factor of this bacterium is toxoflavin, a phytotoxin that acts as an effective electron carrier and generates toxic reactive oxygen species in host cells (Sato et al., 1989; Kim et al., 2004; Suzuki et al., 2004). Biosynthesis and transport of toxoflavin are mediated by the tox-ABCD and toxFGHI gene clusters, respectively, under the positive regulation of the transcriptional regulators ToxJ and ToxR (Kim et al., 2004, 2009; Suzuki et al., 2004; Fenwick et al., 2016). Lipase (Devescovi et al., 2007), flagellum-dependent motility (Kim et al., 2007), and catalase (Chun et al., 2009) also contribute to the pathogenesis of *B. glumae*. Recently, we identified extracellular protease, solely conferred by prtA, as a new virulence factor of this bacterium (Lelis et al., 2019).

Regulation of the major virulence-related functions of *B. glumae* involves multiple regulatory pathways and components for pertinent expression of various virulence genes in a timely manner during colonization and infection processes (Ham, 2013). Quorum-sensing (QS) systems, commonly found in many gram-negative bacteria, are mediated by acyl-homoserine lactone (AHL)-type signalling molecules and the cognate receptors of AHL signal molecules regulate various cellular functions in a cell density-dependent manner (Fuqua et al., 1996). The prototypical system of AHL-mediated QS is the LuxI/LuxR system of *Vibrio fischeri*, in which LuxI and LuxR act as the AHL synthase and the AHL receptor, respectively (Fuqua et al., 1996). In *B. glumae*, the AHL-mediated QS system, composed of the LuxI homolog, TofI, and the LuxR homolog, TofR, plays pivotal roles in regulating known virulence-related functions of this bacterium (Kim et al., 2004, 2007; Devescovi et al., 2007). In particular, ToxR/acylhomoserine lactone (C8-HSL) complex activates the transcription of the toxoflavin activator genes, toxJ and toxR, which further triggers expression of toxoflavin-biosynthesis genes (toxABCD) and transport genes (toxFGHI) via physical binding of ToxR and ToxJ to the regulatory sequences of the genes (Kim et al., 2004). Other virulence factors, including lipase production, flagellum-dependent motility, catalase activity, and extracellular protease activity of *B. glumae*, are substantially compromised in tofI/tofR QS-defective mutants (Devescovi et al., 2007; Kim et al., 2007; Chun et al., 2009; Lelis et al., 2019).

QsmR, an IclR-type transcriptional regulator, was originally identified as a key positive regulator of flagellum biogenesis and flagellum-dependent motility in *B. glumae* (Kim et al., 2007). Recent studies showed that QsmR also exhibits consequential regulatory roles in other virulence factors of *B. glumae*. QsmR activates expression of katG, encoding a catalase known to have a role in protecting *B. glumae* cells from visible light (Chun et al., 2009). Mutation of qsmR also causes a significantly decreased level of toxoflavin (Chen et al., 2015) and extracellular protease (Lelis et al., 2019). Nevertheless, the relationship between QsmR and the tofI/tofR QS system seems to vary among different strains of *Burkholderia*. asmR has been shown to be transcriptionally activated by the tofI/tofR QS in *B. glumae* BGR1 (Kim et al., 2007), but this is not observed in *B. glumae* 336gr-1 (Lelis et al., 2019).

In a previous study, we identified tepR, encoding a group I bacterial enhancer-binding protein (bEBP), as a novel negative regulator of toxoflavin from a forward genetic screening experiment (Melanson et al., 2017). Though not previously investigated in *B. glumae*, group I bEBPs contain an N-terminal response regulator domain for signal perception, a $\sigma^{54}$-binding domain for nucleotide binding, ATP hydrolysis, and interaction with $\sigma^{54}$, and a DNA-binding domain that allows recognition of a specific cis-acting regulatory sequence (Wigneshweraraj et al., 2005; Bush and Dixon, 2012). Group I bEBP can function as a transcriptional activator or a repressor. NtrC is a well-characterized group I bEBP that activates transcription of the glutamine synthetase glnA gene for assimilation of nitrogen in *Escherichia coli* (Carmona and Magasanik, 1996); inactivation of another group I bEBP, luxO, renders increased extracellular protease activity in several *Vibrio* spp. (Zhu et al., 2002; Sultan et al., 2006; Wang et al., 2007; Elgaml et al., 2014).

Here we report the genetic basis of tepR in its negative regulatory functions on toxoflavin and other virulence factors, as well as heat tolerance of *B. glumae*, through transcriptomic and genetic approaches. We also show that tepR positively regulates a previously uncharacterized type VI secretion system of *B. glumae*, which contributes to interspecies competition in this bacterium. A possible working model for the regulatory network involving tepR and the tofI/tofR QS is also proposed in this study.

RESULTS

2.1 General features of the tepR gene

The tepR gene encodes a bEBP protein that contains three functional domains: a response regulator domain, a $\sigma^{54}$-binding domain, and a DNA-binding domain (GenBank ID ACR28150.1; Figure S1). Homologs of TepR were identified in other bacteria through BLASTp ($E$ value $<10^{-5}$; amino acid identity $>30$%). Phylogenetic analysis with the maximum-likelihood method (Figure 1) and comparative alignment (Text S1) of the TepR homologs indicated that TepR is well conserved among bacterial species of Burkholderiaceae.
2.2 | Transcriptome profiling of the ΔtepR and ΔtofI-tofR derivatives of B. glumae

In view of the central regulatory roles of the tofI/tofR QS for the virulence-associated functions of B. glumae, we conducted a transcriptomic analysis comparing the wild-type strain B. glumae 336gr-1 and its ΔtepR and ΔtofI-tofR (deletion of the entire tofI/tofR gene cluster) derivatives to investigate the regulatory functions of tepR and possible linkages between tepR and the tofI/tofR QS. The genome of B. glumae BGR1 (Lim et al., 2009) was used as the reference genome to identify differentially expressed genes (DEGs) of B. glumae 336gr-1 in this study. Total RNA samples were harvested when cultures reached early stationary phase (OD 600 = 1.0). The transcriptome profiling yielded 310 DEGs in ΔtepR and 303 DEGs in ΔtofI-tofR, respectively (Table S1 and Figure S2). A shared subset of 167 genes was differentially expressed in both ΔtepR and ΔtofI-tofR, including 154 genes being significantly up- and down-regulated in ΔtepR and ΔtofI-tofR, respectively (Figure 2a). The largely opposite transcriptomic changes in ΔtepR and ΔtofI-tofR suggested that tepR acted majorly as a negative regulator of genes that were positively modulated by the tofI/tofR QS system.

We recently showed that extracellular protease, solely contributed by prtA (corresponding to bglu_1g16590 of the reference genome B. glumae BGR1), is a new virulence factor of B. glumae, which is under positive transcriptional regulation of the tofI/tofR QS and qsmR (Lelis et al., 2019). In line with this, expression of prtA was significantly less expressed in the ΔtofI-tofR mutant with a 20-fold reduction compared to that in the wild-type 336gr-1. In contrast, expression of prtA in the ΔtepR mutant was 4.6-fold higher than that in the wild-type 336gr-1, suggesting the negative regulatory role of tepR in affecting prtA, and extracellular protease activity of B. glumae. Both groEL (bglu_2g19330) and groES (bglu_2g19340), chaperone protein-encoding genes, were significantly up-regulated in ΔtepR but not differentially expressed in ΔtofI-tofR. The DEGs in ΔtepR and ΔtofI-tofR were further analysed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway/module enrichment analysis. The DEGs that were associated with the “quorum sensing” (bgl02024) pathway were over-represented in both ΔtepR and ΔtofI-tofR (Figure 2g) with q values of 3.4 × 10^{-6} and 3.8 × 10^{-7} respectively (Table S2). The “quorum sensing” pathway of B. glumae includes the tofI/tofR QS-regulated toxoflavin biosynthesis genes (toxABCD), toxoflavin transport genes (toxFGH), and their known regulatory genes (toxJ and toxR). As expected, all the toxoflavin-biosynthesis and -transport genes were expressed at significantly higher levels in ΔtepR but lower levels in ΔtofI-tofR. However, toxJ was expressed 2-fold lower in ΔtofI-tofR but not differentially expressed in ΔtepR. Although toxR was not differentially expressed in either ΔtepR or ΔtofI-tofR under our criteria of DEG, toxR was 2.6-fold decreased in ΔtofI-tofR with a p value of .075, slightly higher than the cut-off p value of .05. The expression pattern of the tox gene cluster was consistent with our previous observation that ΔtepR produced a considerably higher amount of toxoflavin, which
was abolished in ΔtofI-tofR (Chen et al., 2012; Melanson et al., 2017). qsmR, playing positive regulatory roles of several virulence factors of B. glumae, such as toxoflavin, extracellular protease, and flagellum-dependent motility, was significantly more highly expressed in ΔtepR but not differentially expressed in ΔtofI-tofR. Coincident with this, flhF, a flagellum biosynthesis regulator gene and an additional seven flagellum assembly associated genes were significantly up-regulated in ΔtepR (S1 Table). The expression levels of selected genes involved in biosynthesis and regulation of toxoflavin were validated by quantitative reverse transcription PCR (RT-qPCR) (Figure S3). The KEGG enrichment analysis also indicated that tepR, but not the tofI/tofR QS system, positively regulates a previously uncharacterized type VI secretion system (T6SS) gene cluster (corresponding to bglu_1g03850–bglu_1g03990 of the reference genome B. glumae BGR1) (Figure 2 and Table S2), denoted as BgT6SS-1. Despite the overall opposite regulatory roles of tepR and the tofI/tofR QS system in toxoflavin genes and the extracellular protease gene prtA, neither tofI nor tofR was differentially expressed in ΔtepR; tepR was not differentially expressed in ΔtofI-tofR as evidenced by the results from both RNA-Seq (Table S1) and RT-qPCR (Figure S3), suggesting that direct crosstalk between the two regulatory systems at the transcription level is unlikely. However, both tepR and the tofI/tofR QS system differentially regulated multiple LuxR family transcriptional regulators, which are potential autoinducer binding proteins, besides TofR. Among the 23 putative LuxR family transcriptional regulator genes identified, four (bglu_2g06470, bglu_1g16610, bglu_1g23190, and bglu_2g19400) were significantly up- and down-regulated in ΔtepR and ΔtofI-tofR, respectively (Figure S4). Another four LuxR family protein genes (bglu_2g06330, bglu_2g14470, bglu_2g19520, and bglu_1g17030) were uniquely down-regulated in ΔtofI-tofR but not differentially expressed in ΔtepR (Figure S4).

### 2.3 The negative regulatory roles of tepR for toxoflavin, extracellular protease, and flagellum-dependent motility through qsmR

The significantly higher expression of prtA in ΔtepR suggested a possible negative regulatory role of tepR in extracellular protease activity. Indeed, extracellular protease activity, indicated by the clear zone formed surrounding a bacterial colony in a Luria-Bertani (LB)-skim milk plate, was significantly higher in ΔtepR than in the wild-type 336gr-1 (Figure 3a). Consistent with the observed phenotypes in the virulence factors, that is, toxoflavin and extracellular protease, ΔtepR also exhibited higher virulence on rice panicles than its parent strain 336gr-1 (Figure S5). Notably, qsmR, playing crucial positive
regulatory roles in the production of toxoflavin and extracellular protease activity in *B. glumae* 336gr-1. (Chen et al., 2015; Lelis et al., 2019), was significantly more highly expressed in ΔtepR (Figures 2b and 3c). This leads to the hypothesis that the negative regulatory role of *tepR* on toxoflavin and extracellular protease production was *qsmR*-dependent.

To validate this hypothesis, we generated a mutant that was devoid of both *tepR* and *qsmR*, ΔtepR/ΔqsmR. In contrast with ΔtepR, which produced a elevated amount of toxoflavin and extracellular protease, ΔtepR/ΔqsmR did not produce a detectable level of either virulence factor (Figure 4a–d). Although *tepR* did not significantly regulate the expression level of *tofI* or *tofR*, the negative roles of *tepR* on toxoflavin and extracellular protease were still *tofI-tofR* QS-dependent, as suggested by the diminished level of toxoflavin and extracellular protease activity in ΔtepR/ΔtofI-tofR, a dual mutation of both *tepR* and the *tofI-tofR* QS gene cluster (Figure 4e–h). Similar to the phenotypes in toxoflavin and extracellular protease, ΔqsmR exhibited minimal swimming motility, while ΔtepR was hypermotile (Figure 5). This is consistent with the significantly higher expression of the flagellum-associated genes in ΔtepR (Table S1).

### 2.4 The negative regulatory role of *tepR* in heat stress tolerance

As *tepR* negatively affected expression of multiple heat shock protein genes, we measured the tolerance of *B. glumae* strains to heat stress by subjecting cultures to an elevated temperature of 50 °C. Viable cells were counted periodically with 10 min intervals. Although no significant difference of cell survival was observed among the strains after 10 min of the heat treatment, ΔtepR survived at significantly higher rates than 336gr-1 and ΔtofI-tofR at 20 or 30 min (Figure 6a). ΔtofI-tofR was significantly more sensitive to the heat
FIGURE 4 The phenotypes of the tepR and qsmR, tepR, and/or tofI-tofR null mutations in extracellular protease activity and toxoflavin production. (a), (b), (e), and (f) The extracellular protease activities of the wild-type 336gr-1 and its ΔtepR, ΔqsmR, ΔtepR/ΔqsmR, ΔtepR, ΔtofI-tofR, and ΔtepR/ΔtofI-tofR derivatives were determined by (a) and(e) an azocasein-based assay and (b) and(f) the halo zone on a nutrient agar (NA) plate supplemented with 1% skim milk. (c), (d), (g), and (h) Toxoflavin production of the wild-type 336gr-1 and its ΔtepR, ΔqsmR, ΔtepR, ΔtepR/ΔqsmR, ΔtepR, ΔtofI-tofR, or ΔtepR/ΔtofI-tofR derivatives. Error bars represent the standard error of three independent experiments with four replications. Columns with different letters indicate statistically significant differences among the data at p < .05 based on Tukey’s post hoc test. ΔtofI-tofR and ΔtepR/ΔtofI-tofR are indicated as ΔtofI-R and ΔtepR/ΔtofI-R, respectively.
stress condition after 30 min of the treatment (Figure 6a). In contrast, ΔtepR did not exhibit any difference from the wild type in cell death at the stationary phase of cell culture, in which the QS mutant ΔtofI-tofR showed rapid cell death (Figure 6b). These results indicate that tepR negatively affects tolerance of B. glumae to heat stress, whereas the tofI/tofR QS system plays a positive role in this trait.

2.5 | The positive regulatory role of tepR in bacterial interspecies competition through BgT6SS-1

Although tepR acted as a negative regulator of multiple virulence factors and heat stress tolerance, it positively regulated multiple genes (bglu_1g03880-bglu_1g03990) in a putative T6SS, denoted as
BglT6SS-1 (bglu_1g03850-bglu_1g03990). Because the functions of T6SSs, to the best of our knowledge, have not been previously characterized in B. glumae, an alignment between B. glumae BglT6SS-1 and B. thailandensis T6SSs was constructed (Figure S6). Based on the composition, order, and sequence similarity of T6SS genes, B. glumae BglT6SS-1 was closest to B. thailandensis T6SS-1. Notably, inactivation of T6SS-1 in B. thailandensis rendered reduced competition ability of B. thailandensis against several bacteria, that is, Pseudomonas putida, Pseudomonas fluorescens, and Serratia proteamaculans (Schwarz et al., 2010). The competition defect was observed only in a T6SS-1-defective mutant of B. thailandensis but not in mutants of other T6SSs of the same bacterial background. This led to our hypothesis that BglT6SS-1 may play a role in interspecies competition of B. glumae. Among the down-regulated BglT6SS-1 genes in the ΔtepR mutant, tssD (bglu_1g03910) encodes an Hcp-like effector protein (PFam: PF05638) and deletion mutation of tssD is presumed to render defective functions of BglT6SS-1 in B. glumae 336gr-1. To test our hypothesis, we generated the ΔtssD mutant in the wild-type 336gr-1 background and conducted a contact-dependent competition assay of B. glumae strains against Pantoea sp. RSPAM1 (Figure 7). A similar competition defect was observed in ΔtepR, and the competition defect was complemented when tepR was introduced in trans (Figure 7). Taken together, we characterized the function of BglT6SS-1 in interspecies competition of B. glumae, which is positively regulated by tepR.

3 | DISCUSSION

tepR, encoding a bEBP, was recently identified as a negative regulator of toxoflavin, the most important virulence factor of B. glumae (Melanson et al., 2017). Toxoflavin and other known virulence factors of B. glumae are under the tight regulation of the tofI-tofR QS system (Kim et al., 2004, 2007; Devescovi et al., 2007; Lelis et al., 2019). In this study, leveraging transcriptomic and genetic approaches, we uncovered the genetic basis of tepR in orchestrating multiple virulence factors, heat stress tolerance, and interspecies competition in B. glumae.

Transcriptomic profiling of ΔtepR and ΔtofI-tofR revealed an overlapping, though opposite, regulation of multiple genes associated with various biological processes by tepR and the tofI-tofR QS system, respectively. The transcriptomic analysis suggested that the toxoflavin biosynthesis and transport genes and the extracellular protease gene prtA were negatively and positively regulated by tepR and the tofI-tofR QS system, respectively. This is in line with the drastic increase and decrease of toxoflavin production and extracellular protease activity in ΔtepR and ΔtofI-tofR, respectively, compared with the wild type, B. glumae 336gr-1 (Chen et al., 2012; Melanson et al., 2017; Lelis et al., 2019). These results reiterate the essential roles of the tofI-tofR QS system in positively regulating virulence factors in B. glumae and highlight tepR as a novel negative regulator of the virulence functions in this bacterium.

Notably, tepR did not significantly regulate tofI or tofR in B. glumae 336gr-1, at least at the transcriptional level. So how do tepR and the tofI-tofR QS system regulate multiple virulence factors (i.e., toxoflavin and extracellular protease) in the opposite way? In the canonic regulation of toxoflavin by the tofI-tofR QS system, the autoinducer

![Figure 7](image-url)
signal C_10-HSL and its cognate receptor TofR form a complex to activate the expression of toxJ, a LuxR family transcriptional regulator gene, which further induces the toxoflavin biosynthesis/transport genes via TofoxR (Kim et al., 2004, 2009). Consistent with the known regulation cascade, toxJ was significantly down-regulated in the transcriptome of ΔtofI-tofR. Nevertheless, toxJ was not differentially expressed in ΔtepR in the transcriptome analysis in this study. This indicates that tepR does not regulate toxoflavin biosynthesis through this canonic pathway. Instead, tepR negatively regulates qsmR, a positive regulatory gene of flagellum biogenesis, that was also recently shown to play positive regulatory roles in toxoflavin production and extracellular protease activity in B. glumae (Kim et al., 2004; Chen et al., 2015; Lelis et al., 2019). We therefore wondered if tepR regulated these virulence-related traits of B. glumae dependent on qsmR. Indeed, toxoflavin production and extracellular protease activity were abolished in the ΔqsmR/ΔtepR mutant. These results suggest that tepR exerts its negative regulatory roles in these virulence factors, at least in part, through repressing expression of qsmR. fliF, essential for the swimming motility of B. glumae BGR1 (Jang et al., 2014), and additional seven flagellum assembly associated genes were significantly up-regulated in ΔtepR, suggesting a possible indirect regulation of these genes by tepR through qsmR.

We also found that ΔtepR was significantly more tolerant to heat stress compared with the wild-type 336gr-1. Although qsmR positively regulates multiple universal stress protein genes (Kim et al., 2012), none of the qsmR-regulated universal stress protein genes were differentially expressed in ΔtepR (Table S1). This suggests that tepR is likely to modulate heat stress tolerance through a different mechanism. Interestingly, chaperonin genes groEL (bglu_2g19330) and groES (bglu_2g19340) were significantly up- and down-regulated in ΔtepR and ΔtofI-tofR, respectively. GroEL and GroES are known to assist the folding of proteins in response to heat challenge and other environmental stresses in many bacterial organisms (Goloubinoff et al., 1997; Kilstrup et al., 1997; Arsene et al., 2000; Lund, 2009; Vabulas et al., 2010; Kumar et al., 2015). Although expression patterns of groEL and groES correlated with the observed heat tolerance phenotypes of ΔtepR and ΔtofI-tofR, more genetic studies with null mutants of the chaperonin genes are needed to attribute the heat tolerance of B. glumae to GroEL and GroES.

It is important to note that tepR positively regulates a previously uncharacterized T6SS gene cluster, BgT6SS-1, according to the KEGG enrichment analysis in this study. T6SSs are known to contribute significantly to bacterial pathogenesis or bacterial cross-species competition (Schwarz et al., 2010; Records, 2011; Salomon et al., 2015; Lennings et al., 2018). As the ΔtepR mutant was significantly more virulent compared with the wild-type 336gr-1, we hypothesized that BgT6SS-1 was less likely to contribute to the virulence of B. glumae. Both ΔtepR and ΔtssD exhibited a significantly compromised competition capacity against the co-cultured Pantoea sp. RSPAM1, a rice seed-associated bacterium, suggesting that tepR is likely to contribute to interspecies competition in B. glumae against other microorganisms of the same environmental niche through positive regulation of BgT6SS-1. In agreement with our finding, inactivation of T6SS-1 in B. thailandensis, homologous to BgT6SS-1 in B. glumae, renders B. thailandensis significantly more susceptible to other bacterial competitors (Schwarz et al., 2010). As BgT6SS-1 genes were not differentially expressed in ΔtofI-tofR and no statistical competition advantage or defect was observed in ΔtofI-tofR compared with the wild-type 336gr-1, tofI/tofR Q5 is, therefore, unlikely to affect the BgT6SS-1-mediated bacterial interspecies competition. Notably, σ^54 has been shown to be a critical modulator of a T6SS in Vibrio cholerae and binding sites of σ^54 in the gene clusters of T6SSs are predicted in various bacterial organisms (Pukatzki et al., 2006; Bernard et al., 2011). The σ^54-dependent transcriptional regulation obligately requires activation by a bEBP (Buck et al., 2000), and the activation of T6SS in V. cholerae by σ^54 is known to be dependent on the bEBP Vash (Kitaoka et al., 2011), sharing 27% amino acid identity to TepR. Whether or not the positive regulation of B. glumae BgT6SS-1 by TepR is through σ^54 is under investigation. The overall regulatory functions of TepR in B. glumae are reminiscent of LuxO in Vibrio spp., although limited sequence similarity is shared between them (32% amino acid identity). LuxO suppresses luminescence of Vibrio harveyi or Vibrio fischeri that is under the positive regulation of the luxI/luxR QS system (Bassler et al., 1994; Freeman and Bassler, 1999; Miyamoto et al., 2000, 2003). LuxO mutants in several Vibrio spp. exhibit significantly enhanced extracellular protease activity (Zhu et al., 2002; Sultan et al., 2006; Wang et al., 2007; Elgami et al., 2014), resembling the same phenotype in the ΔtepR mutant of B. glumae 336gr-1. Similar to tepR, luxO regulates two T6SSs in V. cholerae (Shao and Bassler, 2012). As the regulatory roles of LuxO appear to be σ^54-dependent (Lilley and Bassler, 2000; Wolfe et al., 2004), we are currently investigating whether the multifaceted regulatory roles of tepR in B. glumae are in part dependent on σ^54.

Taken together, tepR acts as a global regulator of a plethora of biological functions in B. glumae. In particular, tepR represses multiple virulence factors in a qsmR-dependent manner. tepR also positively modulates other biological functions, including heat tolerance and interspecies bacterial competition. We showed that the interspecies competition capacity of B. glumae 336gr-1 is contributed by BgT6SS-1, which is positively regulated by tepR. Based on the results of this study, a working model summarizing the central regulatory roles of tepR in B. glumae is proposed in Figure 8. Further study of tepR in terms of its functional mechanisms in conjunction with the tofI/tofR QS system and other regulatory components will provide more information about the signalling and regulatory pathways involved in the parasitic fitness of B. glumae and related bacterial pathogens.

### 4.1 Experimental procedures

#### 4.1.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S3. B. glumae 336gr-1 and E. coli were grown and maintained following the routine methods described in our previous study (Karki et al., 2012).
4.2 | DNA cloning and amplification

Routine procedures for DNA cloning and amplification were conducted following Sambrook (2001) and the methods described in our previous study (Karki et al., 2012). The primers used for conventional and quantitative PCR are listed in Table S4.

4.3 | Generation of the ΔtepR-2, ΔtepR-3, ΔtepR/tofI/tofR, and ΔtepR/qsmR derivatives of 336gr-1

The ΔtepR stains of B. glumae used in this study were generated following the method described in our previous study (Melanson et al., 2017).

4.4 | Generation of a T6SS-deficient derivative of 336gr-1

To generate a T6SS-deficient derivative of 336gr-1, a 385-bp upstream flanking region and a 345-bp downstream flanking region of tssD (bglu_1g03910 of the reference strain BGR1) were amplified using the primer sets 3910UpF/3910UpR and 3910DWNF/3910DWNR, respectively (Table S4). PCR products were ligated into the PCR cloning vector pSC-A-amp/kan to generate TopoPV3.2 (upstream fragment) and TopoPV4.3 (downstream fragment). TopoPV4.3 was digested with SpeI and XbaI and cloned into pKKSacB to generate pKKSacBT6SSdwn. The upstream fragment was digested with SpeI and cloned into pKKSacBT6SSdwn to generate pKKSacBT6SS. pKKSacBT6SS was transformed into E. coli S17-1::pir competent cells through electroporation followed by conjugation into B. glumae via tri-parental mating using the E. coli HB101 (pRK2013::Tn7) helper strain. A single homologous recombinant was selected on LBagar containing nitrofurantoin and kanamycin. Subsequently, the selected colonies were grown overnight at 30 °C in LB broth without any antibiotics and the cultures were spread on LB agar containing 30% sucrose to select mutants with secondary homologous recombination. Sucrose-resistant colonies were tested for sensitivity to kanamycin, and deletion of tssD was confirmed by PCR using the primers 3910UpF and 3910DWNR.

4.5 | RNA extraction, quality assessment, and sequencing

RNA extraction was conducted following the protocol previously established in our laboratory (Melanson, 2014). Briefly, overnight cultures of three biological replicates of each B. glumae strain were washed and diluted 100 times in fresh LB broth. Diluted cultures were routinely grown until the optical density reached OD₆₀₀ = 1.0. One millilitre of each culture was pelleted and resuspended in 1 ml of TRizol reagent (Ambion Life Technologies). Samples were stored at −70 °C before proceeding. Total RNA was extracted using the Direct-zol RNA MiniPrep Kit with in-column DNase treatment (Zymo...
Research) according to the manufacturer’s instructions. A second DNase treatment was performed using the DNase Treatment and Removal kit (Ambion Life Technologies) to remove any residual DNA contamination. The resultant total RNA samples were sent to the Johns Hopkins University Genetic Resources Core Facility for subsequent RNA-Seq steps including ribosomal RNA depletion, library construction, and sequencing. RNA quality was assessed by a 2100 BioAnalyzer (Agilent Technologies), and the RNA-Seq libraries were prepared with the ScriptSeq v. 2 RNA-Seq Library Preparation Module of the ScriptSeq Complete Kit (Bacteria) (Epicentre). RNA sequencing was conducted on a single lane of Illumina HiSeq 2500, HCS v. 2.2.38, RTA v. 1.18.61 (Illumina, Inc.) to obtain 100 bp paired-end reads.

4.6 | Assembly and annotation of transcriptomes

The quality of the raw reads was evaluated by FastQC v. 0.11.2 (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adaptor sequences and low-quality reads were removed by Trimmomatic v. 0.32 (Bolger et al., 2014). The filtered reads were mapped to the reference genome (the genome of B. glumae BGR1) (Lim et al., 2009) using Bowtie2 v. 2.3.4.3 (Langmead and Salzberg, 2012). Read counts were obtained by HTSeq v. 0.6.1 (Anders et al., 2015). Trimmed mean of M values (TMM)-normalized counts per million (CPM) mapped reads were obtained by edgeR (Robinson et al., 2010). DEGs were determined with a cut-off fold-change and q values of 1.5 and 0.05, respectively.

4.7 | KEGG enrichment analysis

The database of KEGG pathway or module of B. glumae BGR1 (the reference genome used) was downloaded from the KEGG website (https://www.genome.jp/kegg/). The KEGG pathway or module enrichment analysis was conducted on the R package clusterProfiler v. 3.4.4 (Yu et al., 2012), with a cut-off q value of 0.05 adjusted by the Benjamini–Hochberg method.

4.8 | RT-qPCR

Independent RNA samples were extracted following the same procedures as in the RNA-Seq study. cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs). Primers used for RT-qPCR are listed in Table S4. For each reaction, 1 µl of template cDNAs, 7 µl of water, and 1 µl of 10 µM of both forward and reverse primers were mixed with 10 µl of 2 × Power SYBR Green PCR Master Mix (Life Technologies) to perform a 20 µl RT-qPCR on a IQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad). The reaction condition was initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s. Gene fold-changes were estimated by the 2^-ΔΔCt formula (Livak and Schmittgen, 2001) using gyrA as the reference gene. Each reaction had three biological replicates.

4.9 | Toxoflavin quantification

Toxoflavin was quantified following the method described in our previous study (Chen et al., 2012).

4.10 | Protease activity assay

Extracellular protease activity of B. glumae was determined by the method described in our previous study (Lelis et al., 2019).

4.11 | Motility assay

The swimming motility assay was performed at 37 °C in tryptone swim plates (1% tryptone, 0.5% NaCl, and 0.3% agar). Each bacterial strain was grown overnight at 37 °C in LB broth. Bacterial cells were harvested, washed with, and resuspended in fresh LB broth at OD600 = 0.1. Five microlitres of the resuspended cells was inoculated on the centre of a tryptone swim plate, and the swimming activity was determined after 48 hr of incubation.

4.12 | Virulence assay

Virulence of B. glumae on rice panicles was determined following the method described in our previous study (Karki et al., 2012).

4.13 | Heat stress and stationary-phase stress assays

Overnight B. glumae cultures were washed twice and resuspended in phosphate-buffered saline (PBS). Five hundred microlitres of the resuspended cultures was transferred to a waterbath at 50°C. Colony-forming units (cfu) were counted at 10-min intervals using the dilution plating method. The survival rate was calculated as the ratio between cfu/ml after the heat treatment and that before the treatment. To test tolerance of B. glumae to stationary-phase stress, overnight cultures were adjusted to c.10^8 cfu/ml (OD600 = 0.1) in fresh LB broth, and the bacterial population was determined by the series dilution plating method at 6 hr intervals until the population reached late stationary phase.

4.14 | Bacterial interspecies competition assay

The overnight culture of bacterial cells grown in LB broth was washed and resuspended in PBS to OD600 = 0.1 (c.10^5 cfu/ml). Bacterial cells were mixed in 1:1 ratio, and 10 µl of the suspension mixture was
spotted on nutrient agar and incubated for 24 hr at 30 °C. Bacterial cells were then resuspended in 1 ml of PBS and serially diluted. Ten microlitres of each serial dilution of the mixture was spotted on LB agar containing selective antibiotics. Surviving bacterial colonies were counted after 24 hr of incubation at 30 °C. Selective markers used in this assay were nitrofurantoin (100 μg/ml) for *B. glumae* and nalidixic acid (20 μg/ml) for *Pantoea* sp. RSPAM1.

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CONFLICT OF INTEREST

The authors of this manuscript have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

J.P., T.L., and J.H.H. designed the experiments. J.P., T.L., R.C., I.B., and S.O. performed the experiments. J.P., T.L., R.C., and J.H.H. analysed the data. J.P., T.L., and J.H.H. wrote the manuscript. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information for this article. The data have been deposited with links to Sequence Read Archive (SRA) accession number SRR11282890-SRR11282899 in the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra/).

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**SUPPORTING INFORMATION**

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