Dissection of Quorum-Sensing Genes in *Burkholderia glumae* Reveals Non-Canonical Regulation and the New Regulatory Gene *tofM* for Toxoflavin Production

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Abstract

*Burkholderia glumae* causes bacterial panicle blight of rice and produces major virulence factors, including toxoflavin, under the control of the quorum-sensing (QS) system mediated by the *lux* homolog, *tofI*, and the *luxR* homolog, *tofR*. In this study, a series of markerless deletion mutants of *B. glumae* for *tofI* and *tofR* were generated using the suicide vector system, pKKSacB, for comprehensive characterization of the QS system of this pathogen. Consistent with the previous studies by other research groups, *Atof* and *Atorf* strains of *B. glumae* did not produce toxoflavin in Luria-Bertani (LB) broth. However, these mutants produced high levels of toxoflavin when grown in a highly dense bacterial inoculum (> 10^11 CFU/ml) on solid media, including LB agar and King's B (KB) agar media. The *AtofI/AtofR* strain of *B. glumae*, LSUPB201, also produced toxoflavin on LB agar medium. These results indicate the presence of previously unknown regulatory pathways for the production of toxoflavin that are independent of *tofI* and/or *tofR*. Notably, the conserved open reading frame (locus tag: bglu_2g14480) located in the intergenic region between *tofI* and *tofR* was found to be essential for the production of toxoflavin by *tofI* and *tofR* mutants on solid media. This novel regulatory factor of *B. glumae* was named *tofM* after its homolog, *rsaM*, which was recently identified as a novel negative regulatory gene for the QS system of another rice pathogenic bacterium, *Pseudomonas fuscovaginae*. The *AtofM* strain of *B. glumae*, LSUPB286, produced a less amount of toxoflavin and showed attenuated virulence when compared with its wild type parental strain, 336gr-1, suggesting that *tofM* plays a positive role in toxoflavin production and virulence. In addition, the observed growth defect of the *AtofI* strain, LSUPB145, was restored by 1 µM N-octanoyl homoserine lactone (C8-HSL).

Introduction

*Burkholderia glumae*, the primary causal agent of bacterial panicle blight (BPB) of rice, is one of the most important disease problems affecting rice production in the southern United States, including Louisiana, Arkansas and Texas [1]. This rice disease has also been reported from many rice-growing areas around the world, including east Asia, southeast Asia and South America [1]. The optimal temperature range for the growth of *B. glumae* is 38–40°C, but this bacterium can also grow at temperatures as high as 50°C [2]. A typical characteristic of *B. glumae* is the production of the bright yellow phytotoxin, toxoflavin, which is a major virulence factor of this pathogen [3–6].

In *B. glumae*, production of major virulence factors, including toxoflavin, is dependent on the quorum-sensing (QS) system mediated by a pair of LuxI and LuxR homologs, *TofI* and *TofR* [4,7,8]. QS is a cell-to-cell communication mechanism that allows bacterial cells to collectively behave like a multicellular organism. In Gram-negative bacteria, QS systems mediated by LuxI and LuxR-family proteins are involved in a diverse range of bacterial behaviors and traits, including formation of biofilm, production of virulence factors, conjugation, and antibiotic action [9,10]. The LuxI/LuxR system, which is considered the prototype of the QS systems of Gram-negative bacteria, was first discovered in *Vibrio fischeri*, a luminous symbiont in marine animals [11,12]. LuxI-family proteins are synthases that produce N-acyl homoserine lactone (AHL)-type intercellular signal molecules; LuxR-family proteins are cognate receptors that specifically bind to the AHL molecules [13].

Two types of AHL molecules, N-octanoyl homoserine lactone (C8-HSL) and N-hexanoyl homoserine lactone (C6-HSL), are synthesized by the LuxI-family protein of *B. glumae*, *TofI* [4]. It is thought that the LuxR-family protein of *B. glumae*, *TofR*, specifically binds to C8-HSL, and the resultant TofR-C8-HSL complex triggers the production of toxoflavin by activating the transcription of *toxJ*, which has a lux box-like cis element (box) upstream of the coding sequence for the binding of the TofR-C8-HSL complex [4]. Unlike C8-HSL, functions of C6-HSL in *B. glumae* and other *Burkholderia* spp. remain unknown. *ToxJ* encoded by *toxJ* is required for the transcription of *toxR* and *ToxR*, a LysR-type transcriptional regulator, in turn activates the expression of the *toxABCDE* and *toxFGH* operons, which harbor gene clusters...
for toxoflavin biosynthesis and transport, respectively [4]. This regulatory cascade (the TofI/TofR QS system → ToxI → ToxR → toxABCDE and toxFGHE) is considered to be the central regulatory system for the production of toxoflavin, which may allow B. glumae to attack host cells in accordance with its population levels at infection sites [4]. Nevertheless, the genetic functions of tofI and tofR as well as additional components of the QS system governing the expression of bacterial virulence genes in B. glumae are not fully understood.

In this study, a series of deletion mutants deleted in the QS genes, tofI and tofR, were successfully generated from the U.S. virulent strain, 336gr-1 [2], for the further characterization of the QS system and the related global regulatory network in B. glumae. Through the genetic analyses conducted in this study, previously unknown tofI- and/or tofR-independent pathways for the production of toxoflavin were revealed and a new regulatory gene required for these pathways, tofM, was discovered between the tofI and tofR loci.

Materials and Methods

Bacterial Strains, Plasmids, Media and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All the Escherichia coli and B. glumae strains were routinely grown or maintained in LB broth or on LB agar plates [14] at 30°C or 37°C (even though the original definition of LB was corrected by Bertani as 'lysogeny broth' [15], the terms, 'LB broth' and 'LB agar', are used to clearly contrast different growth conditions tested in this study). Bacterial strains grown in liquid media were incubated in a shaking incubator at 200 rpm. LB agar plates amended with 30% sucrose were used to counter-select the recombinant mutants that lost the sucrose-sensitive gene, sacB, through the secondary homologous recombination. The levels of bacterial growth and toxoflavin production in liquid or solid media were determined in four different growth conditions: LB alone, LB with 1 μM C6-HSL (Sigma-Aldrich, St. Louis, MO, USA); LB with 1 μM C8-HSL (Sigma-Aldrich, St. Louis, MO, USA); and LB with both 1 μM C6-HSL and 1 μM C8-HSL. The antibiotics and their working concentrations used in this study were: ampicillin (Amp), 100 μg/ml; kanamycin (Km), 50 μg/ml; nitrofurantoin (Nt), 100 μg/ml; gentamycin (Gm), 20 μg/ml; and tetracycline (Tc), 20 μg/ml.

Recombinant DNA Techniques

Routine DNA cloning and amplification procedures were conducted following standard methods [14]. PCR products used for cloning were purified using the QuickClean 5 M PCR Purification Kit (GenScript, Piscataway, NJ, USA) and cloned into pSC-A-amp/kan using the Strata CloneTM PCR cloning kit (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA of the wild type and mutant strains were extracted using the GenEluteTM Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). Electroporation for transforming E. coli cells was conducted with a GenePulser unit (BioRad Laboratories, Hercules, CA, USA) at 1.5 kV with 20 μF. The bacterial strains and plasmids used in this study are listed in Table 1. All the bacterial strains and plasmids used in this study are listed in Table 2.

DNA Constructs for the Targeted Deletions of tofI, tofM, and tofR

DNA constructs for deletion mutations and deletion mutants of B. glumae generated in this study are listed in Table 1. PCR primers used to create and confirm deletion mutations are listed in Table 2. All deletion mutants generated in this study were obtained through double-crossover homologous recombination in the flanking regions of targeted genes (Figure 1).

To construct pKKSacBΔtofI that was used to create the tofI deletion mutants, a 545-bp region upstream and a 512-bp region downstream of tofI were amplified with the primer sets, tofI-F/tofI-R and tofR-F/tofR-R, respectively (Table 2). The resultant PCR products for these tofI flanking sequences were initially cloned into pSC-A-amp/kan to generate pSCtofIU and pSCtofID. The upstream region of tofI in pSCtofIU was cut with KpnI and BamHI and was then ligated to pLDtofID, cut with the same restriction sites, to generate pLDtofIUD. Because initial attempts to generate a tofI deletion mutant with pLDtofIUD using the tetracycline-resistant gene in pLD55 as a counter-selection marker in the presence of fusaric acid [19] failed, the deletion construct cloned into pLD55 was moved to pKKSacB through the following steps: the 1.1-kb KpnI/XbaI cut fragment from pLDtofIIUD was first ligated to pJP5603, cut with KpnI and XbaI, to generate pJPtofIIUD and increase the choice of restriction sites for the final cloning into pKKSacB; the 1.1-kb SacI cut fragment derived from the native SacI site present 68 bp downstream from the 5’ end of the tofI upstream region cloned into pJPtofIIUD and the SacI site in the polylinker region of the same plasmid was then ligated into pKKSacB, cut with SacI, to obtain pKKSacBΔtofI.

To construct pKKSacBΔtofR that was used to create the tofR deletion mutants, a 426-bp region upstream and an 829-bp region downstream of tofR were amplified with the primer sets, TofR-F/TofR-R and TRD6/TRD3, respectively (Table 2). The resultant
### Table 1. Bacterial strains and plasmids used in this study.

| Strain or Plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **Escherichia coli** | | |
| DH10B | F' araD139 Δ(lac-proAB)7697 ΔlacX74 galU galK rpsL, deaR Δ808lacZΔM15 endA1 supE44 recA1 mcrA (ΔmcrBC) | [28] |
| DH5α | F' endA1 hsdR17 (rK·mK·) supE44 thi-1 Δ (recA1 gyrA96 relA1 deaR Δ [lacZYA-argF-U169])-80lacZΔM15 | [28] |
| S17-1pir | recA thi pro hsdR (res- mod+)JBR4:2-Tc-Mu-Km::Th7) : pir phage lysogen, Sm', Tp' | [29] |
| **Burkholderia glumae** | | |
| 336gr-1 | Wild type strain isolated from diseased rice in Crowley, Louisiana, USA | This study |
| LSUPB139 | A ΔtofR-tofR derivative of 336gr-1 | This study |
| LSUPB145 | A Δtof derivative of 336gr-1 | This study |
| LSUPB169 | A ΔtofR derivative of 336gr-1 | This study |
| LSUPB201 | A ΔtofR/ΔtofR derivative of 336gr-1 | This study |
| LSUPB286 | A ΔtofM derivative of 336gr-1 | This study |
| LSUPB292 | A ΔtofR/ΔtofR derivative of 336gr-1 | This study |
| LSUPB293 | A ΔtofR/ΔtofR derivative of 336gr-1 | This study |
| LSUPB294 | A ΔtofR/ΔtofR derivative of 336gr-1 | This study |
| **Chromabacterium violaceum** | | |
| C. violaceum CV026 | A biosensor that produces a purple pigment in the presence of AHL molecules | [20] |
| **Plasmid** | | |
| pBBR1MCS-2 | A broad host range cloning vector, RK2 ori, lacZα, KmR | [30] |
| pBBR1MCS-5 | A broad host range cloning vector, RK2 ori, lacZα, GmR | [30] |
| pBTofIM | A subclone of pBTofIMR for the 2,808-bp tofR/tofM region inserted into pBBR1MCS-5 at the BglII and SacI sites, GmR | This study |
| pBTofIMR | A subclone of pCos808 for the 3,670-bp tofI/tofM/tofR region inserted into pBBR1MCS-5 at the EcoRI and SacI sites, KmR | This study |
| pBTofMR | A tofM clone in pBBR1MCS-5, GmR | This study |
| pBTofRM | A subclone of pBTofIMMR for the 1,925-bp tofR/tofM region inserted into pBRR1MCS-2 at the EcoRI and PvuII sites, KmR | This study |
| pCos808 | The cosmid clone harbouring tofI, tofM and tofR, AmpR | This study |
| pJP5603 | A suicide vector, R6K γ-ori, RP4 oriT, lacZα, KmR | [31] |
| pJTofIUD | A subclone of pLDTofIUD containing the upstream and downstream flanking regions of tofI in pGP5603, KmR | This study |
| pKKSacB | A suicide vector; R6K γ-ori, RP4 oriT, sacB, KmR | [Ham and Barapha, unpublished] |
| pKKSacBΔtofI | A subclone of pJTofIUD for the upstream and downstream flanking regions of tofI in pKKSacB, KmR | This study |
| pKKSacBΔtofMR | A subclone of pLDTofIUD carrying the downstream flanking regions of tofI and tofR in pKKSacB, KmR | This study |
| pKKSacBΔtofM | A plasmid carrying the upstream and downstream flanking regions of tofI in pKKSacB, GmR | This study |
| pKKSacBΔtofR | A subclone of pKKTofMU for the upstream and downstream flanking regions of tofR in pKKSacB, KmR | This study |
| pKKTofMU | A subclone of pSCTofMU for the upstream flanking region of tofM in pKKSacB, KmR | This study |
| pKKTofRD | A subclone of pSCTofRD for the downstream flanking region of tofR in pKNOCK-Km, KmR | This study |
| pKKTofRU | A subclone of pSCTofRU for the upstream flanking region of tofI in pKTofRD, KmR | This study |
| pKNOCK-Km | A suicide vector; R6K γ-ori, RP4 oriT, KmR | [32] |
| pLD55 | A suicide vector; F1 ori, R6K γ-ori, RP4 oriT, lacZα, AmpR, TcR | [19] |
| pLDTofID | A subclone of pSCTofID for the downstream flanking region of tofI in pLD55, AmpR, TcR | This study |
| pLDTofORD | A subclone of pLDTofID for the downstream flanking region of tofI in pLD55, AmpR, TcR | This study |
| pLDTofIUD | A subclone of pSCTofIUD for the upstream flanking region of tofI in pLDTofID, AmpR, TcR | This study |
| pRK2013::Tn7 | A helper plasmid; ColE1 ori | [16] |
| pSC-A-amp/kan | A blunt-end PCR cloning vector; F1 ori, pUC ori, lacZα, KmR, AmpR | Stratagene |
| pSCTofID | A clone of the 512 bp downstream flanking region of tofI in pSC-A-amp/kan, AmpR, KmR | This study |
| pSCTofIU | A clone of the 545-bp upstream flanking region of tofI in pSC-A-amp/kan, AmpR, KmR | This study |
| pSCTofM | A clone containing the 986-bp region of tofM and its upstream region, AmpR, KmR | This study |
| pSCTofMD | A PCR clone of the 412-bp downstream flanking region of tofM in pSC-A-amp/kan, AmpR, KmR | This study |
PCR products were cloned into pSC-A-amp/kan to generate pSCtofRU and pSCtofRD, respectively. The downstream region of tofR in pSCtofRU was removed using the *BamHI* site in the primer and the *PstI* site in the polylinker region of the plasmid and subsequently ligated to pKNOCK-Km, cut with *BamHI* and *PstI*, to get pKKnM. The upstream region of tofR in pSCtofRU, obtained from *SpeI* and *BamHI* digestion, was then subsequently ligated into pKKnM using the same restriction sites, to create the pKKnM. Finally, the *SpeI*-cut 1.3-kb DNA fragment containing the recombinated flanking regions of tofR from pKKnM was cloned into the *SpeI* site to obtain pKKSacBtofMU. The downstream region of tofR, obtained by *KpnI* digestion of pKKtofRUD was cloned into pKKSacBtofMU, cut from pKKtofMU by *SpeI* and *XbaI*, then ligated to pKKSacBtofMU, cut with *SpeI* and *XbaI*, to obtain pKKSacBtofMR.

To construct pKKSacBtofMR that was used to create the tofM deletion mutants, a 433-bp region upstream and a 412-bp region downstream of tofM were amplified with the primer sets, UPHP-FP/*tofM* and DWP-RP/*tofM*, respectively (Table 2). The amplified PCR products were initially cloned into pSC-A-amp/kan to generate pKKnM and pKKnM, respectively. The downstream region of tofM, obtained by *KpnI* and *BamHI* digestion and subsequently ligated into pLDtofIDR, cut with *KpnI* and *BamHI*, to get pLDtofIDRD. The 1.3-kb DNA fragment that resulted from the *SpeI* digestion of pLDtofIDRD was then ligated to the *SpeI*-cut pKKSacBtofMU to obtain the final deletion construct, pKKSacBtofMR.

DNA Constructs for the Complementation of the QS Mutants

A cosmid library of the *B. glumae* 336gr-1 genome was screened with the primers, tofI-Jh1 and tofI-Jh2, to identify the cosmid clone that contains tofI. The cosmid clone, pCos808, was identified to contain tofI as well as tofR and tofM. pBBtofIMR, which contains tofI, tofM, and tofR, was constructed by cloning a 3,670-bp DNA fragment containing tofI, tofM, and tofR from Cos809 into pBRRMCS-2 using the *EcoRI* and *SacI* sites. pBBtofIM was generated by subcloning the 2,808-bp tofI/tofM region of pBBtofIMR into pBRRMCS-5 using *BglII* and *SacI* sites. pBBtofMK was constructed by subcloning the 1,925-bp tofR/tofM region of pBBtofIMR into pBRRMCS-2 using *EcoRI* and *PvuII* sites. For pBBtofMR, a 986-bp region that includes tofM was amplified using the primers orf1-CT-F and orf1-CT-R (Figure 1 and Table 2). The PCR products were initially cloned into the pSC-A-amp/kan vector following the manufacturer’s protocol to generate pSCtofM. Then, the tofM region of pSCtofM was subcloned into pBRRMCS-5 using *SpeI* and *HindIII* sites to get pBBtofM.

For complementation, each of these constructs was introduced into the appropriate *B. glumae* strain through triparental mating [16].

### AHL Production Assays

*Cronobacter violaceum* CV026, which produces the purple pigment, violacein, in the presence of AHL molecules [20], was used as a biosensor to determine the AHL production by *B. glumae*. The AHL production assay was performed following the procedure used by Kim et al. [24] with some modifications. Briefly, the supernatant fraction of an overnight culture of each *B. glumae* strain grown in LB broth at 37°C obtained after centrifugation was extracted with an equal volume of ethyl acetate, air-dried in a fume hood, and the residue dissolved in 1% volume of sterile distilled deionized water. Then, 20 μl of each culture extract was applied to the cells of *C. violaceum* CV026 immediately after they were inoculated on a LB agar plate. The production of the purple pigment by this biosensor strain was observed after 48 h incubation at 30°C.

### Quantification of Bacterial Growth

To quantify bacterial growth in liquid and solid media, an equal amount of overnight culture per volume of medium was applied to liquid and solid media (~10⁵ cell/ml medium). For solid medium, 12.5 μl of an overnight culture were spread on an LB agar plate containing approximately 12.5 ml of LB agar. For liquid medium, 3 μl of the same overnight culture were added to 1 ml LB broth. After incubation at 37°C for 24 h, bacterial growth was determined by measuring the absorbance of the bacterial culture suspension at 600 nm (OD600). Overnight cultures in LB broth were measured directly. Cultures grown on LB agar plates were resuspended in 12.5 μl of fresh LB broth and then measured for OD600.

### Quantification of Toxoflavin Production

Toxoflavin production by each strain of *B. glumae* was quantified following a previously established method [4] with some modifications for cultures grown in both liquid and solid media. For bacteria grown on LB broth, toxoflavin present in the supernatant obtained from the centrifugation of 1 ml of culture was extracted with 1 ml of chloroform. Following centrifugation, the chloroform fraction was transferred to a new microtube and air-dried in a fume hood. The residue in the microtube was dissolved in 1 ml of 80% methanol. For bacteria grown on LB agar, bacterial cells were removed from the surface of the agar and the remaining agar containing the diffused toxoflavin was cut into small pieces with a razorblade. The chopped agar was then mixed with chloroform in 1:1 (w/v) ratio for toxoflavin extraction and the chloroform fraction was filtered through filter paper and collected in a new microtube. Chloroform was evaporated and culture filtrate residue was dissolved in 80% methanol as previously described. The absorbance of each sample was measured at 393 nm to determine the relative amount of toxoflavin [21].

### Virulence Tests for *B. Glumae*

The onion assay system that was previously used to determine the virulence of *Burkholderia cenocepacia* [22] and *B. glumae* [18,23] was adopted in this study with minor modifications. Briefly, the fleshy scales of yellow onions were cut into pieces (~2×4 cm) with
a sterile razorblade and a 2 mm-slit was made in the center of each onion piece with a sterile micropipette tip. Two microliters of bacterial suspensions made from cultures grown on a LB agar plate, suspended in 10 mM MgCl2 and adjusted to $5 \times 10^7$ CFU/ml, were applied to the slit on each piece of onion scale. The inoculated onion scales were incubated in a moist chamber at 30°C for 72 h. The virulence level of each B. glumae strain was assessed by measuring the area of maceration on each onion scale. Virulence of B. glumae strains in rice was tested following a previously established method [23].

Results

Generation of a Series of Markerless Deletion Mutants of tofI and tofR

Mutant derivatives of B. glumae 336gr-1 with deleted tofI, tofR, or the entire tofI-tofR region, including the intergenic region, were generated using the pKKSacB system (Ham and Barphagha, unpublished), following the procedures described in the Materials and Methods section (Table 1 and Figure 1). Genetic confirmation of the deletion mutants, LSUPB145 ($\Delta$tofI), LSUPB169 ($\Delta$tofR), and LSUPB139 ($\Delta$tofI-tofR), was performed using PCR and primers corresponding to the DNA sequences flanking each deleted region (Figure 2 and Table 2). The size of the PCR products amplified from each mutant was the same as that of the PCR products amplified from the DNA construct used for the corresponding deletion mutation, and the size difference of the PCR products between the wild type and each mutant was matched to the predicted size of the deleted DNA sequence (Figure 2).

Mutations in tofI and tofR were also confirmed with the biosensor strain, C. violaceum CV026, which produces the purple pigment, violacein, in the presence of AHL compounds, including C6-HSL and C8-HSL [20]. The culture extract of the wild type strain, 336gr-1, caused the production of violacein by the biosensor, while that of the deletion mutants did not (Figure 2D), indicating that these mutants did not produce the AHL molecules required for QS. Likewise, none of the mutants produced toxoflavin in either LB agar or LB broth (Figures 2E and 3). These results were consistent with the previous study by Kim et al.
which showed the dependence of toxoflavin production by B. glumae on tofI and tofR.

Restoration of Bacterial Growth and Toxoflavin Production in the ΔtofI Strain, LSUPB145, by C8-HSL

All the QS mutants produced little toxoflavin compared to the wild type in both liquid and solid media (Figure 3). If 1 μM C8-HSL was added to the media, the ΔtofI mutant, LSUPB145, regained the ability to produce toxoflavin, but the ΔtofR mutant, LSUPB169, and the ΔtofI-ΔtofR mutant, LSUPB139, did not (Figure 3). Patterns of toxoflavin production by mutant strains in the presence of exogenous synthetic AHL compounds were similar in both liquid and solid media (Figure 3). In both growth conditions, LSUPB145 appeared to produce more toxoflavin than the wild type 336gr-1 in the presence of 1 μM C8-HSL (Figure 3).

According to the statistical analysis using a two-sample t-test, the toxoflavin production in 336gr-1 and LSUPB145 was significantly different from each other in solid media (T value = 2.3.97, P value = 0.0166) but not in liquid media (T value = 2.1.95, P value = 0.1888).

In addition, the QS mutant strains showed reduced growth when compared to the wild type in both liquid and solid media after 24 h incubation at 37°C (Figures 4 and S1). ANOVA and post hoc LSD tests validated that the observed growth reduction of all the three QS mutants in both types of medium condition was statistically significant (not shown). The difference in bacterial growth between the wild type and the QS mutants appeared to be greater in solid media than in liquid media (Figure 4). Addition of C8-HSL to both liquid and solid media restored the growth of the ΔtofI strain, LSUPB145, to the wild type level, but did not have any effect on the growth of the other QS mutants or the wild type strain (Figures 4 and S1). C6-HSL did not affect the growth of any strain tested (Figures 4 and S1).

Toxoflavin Production of ΔtofI and ΔtofR Derivatives of the Wild Type Strain, 336gr-1, at High Culture Density on LB Agar

ΔtofI and ΔtofR mutants, LSUPB145 and LSUPB169, respectively, produced toxoflavin when grown on solid media after inoculation with the streaking method using an inoculation loop (Figure 5A). The ΔtofI-ΔtofR strain, LSUPB139, on the other hand, did not produce any detectable toxoflavin in the same condition (Fig. 5A). Even though LSUPB145 and LSUPB169 produced less amounts of toxoflavin than the wild type, 336gr-1, did in this growth condition (Figure 5A), their phenotypes were strikingly different from those shown in LB broth (Figure 3A) or LB agar inoculated with the spreading method (Figure 3B). Similar results were observed in tests with other types of solid media, including King’s B agar [24] (data not shown). In an onion assay established to indirectly determine the virulence of B. glumae [25], LSUPB145
and LSUPB169, but not LSUPB139, were able to cause comparable or larger maceration symptoms on onion bulb scales in comparison with the wild type (Figure 5B). Inoculums prepared from the cultures in LB broth and LB agar showed similar results (data not shown).

Identification of a New Regulatory Gene, tofM, in the Intergenic Region between tofI and tofR

Based on the observation mentioned above, we speculated that toxoflavin could be produced in a tofI- or tofR-independent manner at certain growth conditions but could not be produced without both tofI and tofR. To verify this notion, a ΔtofI/ΔtofR double deletion mutant (ΔtofI/ΔtofR), LSUPB201, was generated through consecutive deletions of tofI and tofR and its phenotype in toxoflavin production was tested in various conditions. Unlike the ΔtofI/ΔtofR strain LSUPB139, the ΔtofI/ΔtofR mutant LSUPB201 still produced toxoflavin on LB agar medium when it was inoculated with the streaking method (Figures S2 and 6B). The only difference between LSUPB139 (ΔtofI/ΔtofR) and LSUPB201 (ΔtofI/ΔtofR) was the presence of the intergenic region between tofI and tofR (Figure 1), suggesting that unknown genetic element(s) present between tofI and tofR may be responsible for the tofI and tofR-independent production of toxoflavin. According to the annotated whole genome sequence of B. glumae BGR1 (NCBI Reference Sequence: NC_012721.2), the coding sequences of tofI (locus tag: bglu_2g14490) and tofR (locus tag: bglu_2g14470) are 612 bp- and 720 bp-long, respectively, and are separated by a region of DNA 799 bp in length that includes a single ORF (locus tag: bglu_2g14480) that is divergently transcribed from tofR (Figure 1). The deduced amino acid sequence of this ORF showed

Figure 2. PCR products from diagnostic PCRs used to confirm deletion mutations in Burkholderia glumae and N-acyl homoserine lactone (AHL) signal production and toxoflavin production of deletion mutants. (A) PCR products amplified from primers, TofI(H)F and TofI(H)R, to confirm the tofI deletion in LSUPB145. Template DNA for each lane is as follows: 1, pKKSacBDtofI; 2, genomic DNA of B. glumae 336gr-1; and 3, genomic DNA of B. glumae LSUPB145. (B) PCR products amplified with primers, TofR(H)F and TofR(H)R, to confirm the tofR deletion in LSUPB169. Template DNA for each lane is as follows: 1, pKKSacBDtofR; 2, genomic DNA of B. glumae 336gr-1; and 3, genomic DNA of B. glumae LSUPB169. (C) PCR products amplified with primers, TofI(H)F and TofR(H)R, to confirm the tofI-tofR deletion in LSUPB139. Template DNA for each lane is as follows: 1, pKKSacBDtofIMR; 2, genomic DNA of B. glumae 336gr-1; and 3, genomic DNA of B. glumae LSUPB139. M indicates the 1 kb Plus DNA ladder (Invitrogen, Santa Clara, CA, USA) used as a marker. (D) Violacein production, shown as a purple pigment, by the biosensor, Chromobacterium violaceum CV026, in the presence of the culture extracts of the B. glumae strains, 336gr-1, LSUPB145, LSUPB169, and LSUPB139. Photo was taken 48 h after application of bacterial culture extracts on C. violaceum CV026 inoculated onto a LB agar plate. (E) Toxoflavin production, shown as a yellow pigment, in the LB broth by B. glumae strains, 336gr-1, LSUPB145, LSUPB169, and LSUPB139. Photo was taken after 24 h incubation at 37°C. doi:10.1371/journal.pone.0052150.g002
22.4% identity to that of RsAM in Pseudomonas fuscovaginae [25] and was found to be highly conserved among Burkhoderia spp. (Table 3 and Figure S3). The DNA sequence of the tofI-tofR intergenic region of B. glumae 336gr-1 was identical to that of B. glumae BGR1.

To determine the function of this ORF, deletion mutations of this ORF were made in strains with the genetic backgrounds of DtofI and DtofR, as well as the wild type background, generating LSUPB201, LSUPB292, and LSUPB286, respectively. The toxoflavin production by LSUPB286 (DtofM) was not significantly different from that by the wild type in both LB broth and LB agar conditions at 37°C (Figure 6A). However, this mutant produced a less amount of toxoflavin when compared to the wild type at 37°C and this tendency was more obvious when the bacteria were grown on LB agar medium (Figure 6A). Moreover, the same deletion in the DtofI or DtofR backgrounds resulted in an almost complete loss of the ability to produce toxoflavin, indicating that this ORF is required for the normal production of toxoflavin (Figure 6B). Thus, this ORF was considered as a functional gene and named as tofM, after rsaM due to the sequence homology and similarity in genetic location between luxI and luxR homologs [25] (Figure S3).

Complementation with the tofM clone, pBBtofM, restored toxoflavin production by the ΔtofM strain, LSUPB289 (Figures S4B, S4C, and S5). However, complementation with this tofM clone did not restore the production of toxoflavin on LB agar by LSUPB294 (ΔtofI/ΔtofM), LSUPB292 (ΔtofR/ΔtofM), LSUPB293 (ΔtofI/ΔtofM/ΔtofR), or LSUPB139 (ΔtofI/ΔtofR) (Figure S5). Complementation with pBBtofRM, which contains tofR and tofM, restored the toxoflavin-deficient phenotype of the ΔtofR/ΔtofM strain, LSUPB292, but did not restore the tofI-independent production of toxoflavin in LSUPB293 (ΔtofI/ΔtofM/ΔtofR) and LSUPB139 (ΔtofI/ΔtofR) (Figure S5). Complementation with pBBtofIM, which contains tofI and tofM, did not restore the production of toxoflavin in the ΔtofI/ΔtofM mutant, LSUPB294.
Furthermore, complementation with pBBtofIMR, which contains tofI, tofM, and tofR, restored the production of toxoflavin in LSUPB293 (ΔtofI/ΔtofM/ΔtofR), LSUPB139 (ΔtofI-ΔtofR), LSUPB201 (ΔtofM), and LSUPB286 (ΔtofI/ΔtofM) (Figures S4A and S5). In this test, LSUPB139 (ΔtofI-ΔtofR) caused few visible symptoms, indicating that tofI, tofR, and tofM are collectively required for the pathogenicity of B. glumae in rice (Figure 7).

**Discussion**

The QS system mediated by the TofI AHL synthase and the TofR AHL receptor is known to be a central regulatory element that governs the expression of the major virulence factors of *B. glumae*, including toxoflavin [4,8], lipase [7], and flagella [8]. In this study, a series of tofI, tofM, and tofR mutants were generated to dissect the function of each of these QS components in the
production of toxoflavin in *B. glumae*. LSUPB145 (∆tofI) and LSUPB169 (∆tofR) produced significantly reduced amounts of toxoflavin compared with the wild type strain, 336gr-1 (Figures 2, 3, and S4A). In addition, the ability of LSUPB145 to produce toxoflavin was restored by the addition of 1 μM C8-HSL, but not C6-HSL (Figure 3). These results were consistent with previous studies with another *B. glumae* strain, BGR1, which demonstrated the dependency of toxoflavin production on the TofI/TofR QS system and C8-HSL [4,8]. Although TofI synthesizes both C6-HSL and C8-HSL as major products [4], the role of C6-HSL is still unknown. Notably, the tofI deletion mutant, LSUPB145, produced higher amounts of toxoflavin compared to the parental strain, 336gr-1, in the presence of 1 μM C8-HSL (Figure 3). This pattern was more obvious in LB agar than in LB broth (Figure 3B). This result strongly suggests that tofI is involved in an unknown activity that suppresses the function of C8-HSL in toxoflavin production.

Intriguingly, even though AHL signals were not produced by either the ∆tofI or the ∆tofR mutant (data not shown), both mutants were able to produce high levels of toxoflavin when inoculated with the streaking method on the LB (Figure 5) or KB agar media (data not shown). Further, LSUPB201, which has deletions of both tofI and tofR, also produced considerable amounts of toxoflavin on solid media (Figures 6 and S2). The tofI, tofR and tofI/tofR mutants generated via different approaches, including transposon mutagenesis and homologous recombination, produced phenotypes similar to those of the ∆tofI, ∆tofR, and ∆tofI/∆tofR strains, indicating that the observed toxoflavin production by tofI, tofR, and tofI/tofR mutants is not an artifact (data not shown). Additionally, significant growth defects observed with the QS mutants suggest that the TofI/TofR QS system controls the bacterial genes required for optimal bacterial growth.

We speculated that the deviated phenotypes of LSUPB145 (∆tofI) and LSUPB169 (∆tofR) in toxoflavin production on solid media (Figures 5 and S2) may be due to other unknown QS signaling molecules (ToFM) that interact with the QS circuitry. To test this hypothesis, we conducted a series of experiments to determine the effect of ToFM on toxoflavin production. Consistent with our previous observations, the ToFM mutant, LSUPB139, produced significantly higher amounts of toxoflavin compared to the parental strain, 336gr-1 (Figures 5 and S2). This result suggests that ToFM plays a crucial role in regulating toxoflavin production in *B. glumae*.
media dependent on different methods of inoculation might be due to the differences in bacterial concentration of the initial inoculum. To test this hypothesis, an overnight culture (~10^9 CFU/ml) of LSUPB145 grown in LB broth was inoculated on LB agar with the streaking method, while a concentrated bacterial suspension (~10^11 CFU/ml) of the same strain was inoculated on LB agar with the spreading method. When an overnight culture (~10^9 CFU/ml) of LSUPB145 was inoculated on LB agar plates with the streaking method, the bacterial cultures frequently failed to produce toxoflavin but occasionally (with about 30% chance) produced toxoflavin (data not shown). In contrast, when a concentrated bacterial suspension (~10^11 CFU/ml) was inoculated on LB agar plates with the spreading method, the bacterial cultures frequently failed to produce toxoflavin but occasionally (with about 30% chance) produced toxoflavin (data not shown). In both inoculation conditions, the chance to produce toxoflavin increased as the bacterial concentration of the initial inoculum was higher (data not shown). These observations suggest that both initial concentration of bacterial inoculum and method of bacterial inoculation are critical factors for the production of toxoflavin on solid media. Based on the observed toxoflavin production by the tofI, tofR and tofI/tofR mutants at certain growth conditions, we speculated that *B. glumae* possesses alternative regulatory pathway(s) for the production of toxoflavin in the absence of TofI and TofR. Because the ΔtofI-tofR mutant, LSUPB139, did not produce toxoflavin in any growth condition tested (Figures 2, 3, 5, S2, and S4A), the intergenic region between tofI and tofR was thought to contain at least one regulatory gene that is responsible for toxoflavin production and independent of tofI and tofR. Indeed, a putative gene divergently transcribed from tofR was found to be involved in the production of toxoflavin and deletion of tofM in the wild type background caused a significant reduction in toxoflavin production (Figures 6A and 7). Toxoflavin production of the ΔtofM strain, LSUPB286, was restored to wild type levels following complementation with the tofM clone, pBBtofM (Figures S4B, S4C, and S5). Nevertheless, complementation of the mutants with functional clones of the mutated genes was frequently unsuccessful (Figure S5), implying that the accurate balance of gene expression based on the correct genomic position and gene dosage of tofI, tofM and tofR is critical for the regulation of toxoflavin production by these genes. In this regard, it is noteworthy that the ΔtofM mutant was complemented by a tofM clone carrying tofM only (pBBtofM), but not by tofM clones carrying additional genes (pBBtofRM and pBBtofIMR); likewise, the ΔtofR and ΔtofIMR mutants were complemented only by pBBtofRM and pBBtofIMR, respectively (Figure

| Locus_tag/Gene | Protein ID (Accession #) | Organism | Identity (similarity) |
|----------------|--------------------------|----------|----------------------|
| bglu_2g14480   | YP_002909042.1           | *Burkholderia glumae* BGR1  | 100%                 |
| bgla_2g11060   | YP_004349067.1           | *B. gladioli* BSR3           | 80.0% (88.7%)        |
| bgla_1p1750    | YP_004362596.1           | *B. gladioli* BSR3           | 23.7% (35.3%)        |
| BACMC1869     | YP_002234480.1           | *B. cenocepacia* J2315      | 52.9% (59.2%)        |
| Bcenmc03_5575  | YP_001779190.1           | *B. cenocepacia* MCO-3       | 52.2% (59.2%)        |
| Bcen_3642      | YP_623507.1              | *B. cenocepacia* AU 1054     | 52.2% (59.2%)        |
| Bmul_3970      | YP_001583945.1           | *B. multivorans* ATCC 17616  | 55.6% (63.4%)        |
| Bam_4117       | YP_776004.1              | *B. ambifaria* AMMD          | 52.9% (59.9%)        |
| BamMC406_4582  | YP_001811254.1           | *B. ambifaria* MC40-6        | 51.6% (58.6%)        |
| BamMC406_5824  | YP_001815818.1           | *B. ambifaria* MC40-6        | 28.0% (38.5%)        |
| Bcep1808_5261  | YP_001117673.1           | *B. vienniensis* G4          | 52.2% (61.1%)        |
| Bcep18194_B1051| YP_371809.1              | *Burkholderia* sp. 383       | 51.6% (59.9%)        |
| BTH_I1111     | YP_439707.1              | *B. thailandensis* E264      | 51.3% (65.8%)        |
| BURPS668_A1294| YP_001062291.1           | *B. pseudomallei* 668        | 50.3% (63.1%)        |
| BPSS0886      | YP_110895.1              | *B. pseudomallei* K96243     | 49.7% (62.4%)        |
| BWA1346       | YP_105962.1              | *B. mallei* ATCC 23344       | 49.7% (62.4%)        |
| rsaM           | CB67624.1/RsaM           | *Pseudomonas fuscovaginae* UPB0736 | 22.4% (37.2%) |
| BURPS106A_A1576| YP_001075610.1           | *B. pseudomallei* 1106a      | 32.5% (43.5%)        |
| BURPS1068_0414| ZP_04810916.1            | *B. pseudomallei* 1106b      | 32.5% (43.5%)        |
| BURPS668_A1657| YP_001062653.1           | *B. pseudomallei* 668        | 32.5% (43.5%)        |
| GBP346_B0905  | EEP50658.1               | *B. pseudomallei* MSHR346    | 32.5% (43.5%)        |
| BPSS1179      | YP_111192.1              | *B. pseudomallei* K96243     | 28.7% (38.9%)        |
| BURPS1710A_A0737| ZP_04955066.1            | *B. pseudomallei* 1710a      | 17.1% (23.2%)        |
| BURPS1710B_A0144| YP_335303.1              | *B. pseudomallei* 1710b      | 17.1% (23.2%)        |
| BTH_I11228    | YP_439424.1              | *B. thailandensis* E264      | 28.2% (41.2%)        |
| Bam_6054      | YP_777932.1              | *B. ambifaria* AMMD          | 24.2% (33.3%)        |
| BamMC406_5825 | YP_001815819.1           | *B. ambifaria* MC40-6        | 11.5% (20.2%)        |

Table 3. TofM homologs in *Burkholderia* spp. and *Pseudomonas fuscovaginae*.
We do not know why the ΔtofIM mutant could not be complemented by any clones carrying both tofI and tofM, including pBBtofIM (Figure S5).

Taken together, these results indicate that tofM is a positive regulator for toxoflavin production. When B. glumae is grown in liquid media or on solid media after inoculation with the spreading method, TofM may supplement the regulatory function of the TofI/TofR QS in the production of toxoflavin. When B. glumae is grown on solid media after inoculation with the streaking method, however, TofM may cause the TofI/TofR QS-independent production of toxoflavin. Even though TofM is likely a key regulatory component of the tofI- and tofR-independent pathway(s) for toxoflavin production, additional regulatory components required for the production of toxoflavin in the absence of tofI or tofR have been identified and are currently being analyzed (Chen and Ham, unpublished).

Even though tofM was identified as a positive regulator for toxoflavin production in this study, its homolog, rsaM, was first reported as a novel negative regulator for the QS systems of another rice pathogenic bacterium, P. fuscovaginae [25]. Nevertheless, rsaM seems to exert positive functions for virulence as well because an rsaM mutant of P. fuscovaginae showed attenuated virulence in rice [25]. Both tofM and rsaM are present in the intergenic region of luxI and luxR homologs and are oriented divergently from the luxR homologs (Figure S3). Recent studies on Pseudomonas spp. including P. aeruginosa, P. putida, and P. fuscovaginae.
revealed that rsaL and rsaM, present in the intergenic regions of luxI and luxR homologs, act as negative regulators controlling the homeostasis of AHL levels [26]. In this study, positive function of tofM in virulence was observed (Figure 7), however, repressive action of tofM on the AHL-mediated QS was somewhat ambiguous in the AHL-detection assay using the biosensor C. violaceum CV026 (Figure S6). The biosensor strain treated with the culture filtrate of the tofM mutant, LSUPB286, showed a stronger purple color than that treated with the culture filtrate of the wild type, 336gr-1 (Figure S6). However, this phenotype of LSUPB286 suggesting a negative role of tofM in the AHL-mediated QS could not be complemented by the tofM clone, pBBtofM. Quantitative analyses to precisely determine the roles of tofM in the expression of tofI and tofR, as well as other virulence genes, of B. glumae and in the production of AHL compounds are currently being conducted (Chen and Ham, unpublished).

A database search for tofM revealed that tofM homologs are conserved in many Burkholderia spp. (Table 3 and Figure S3), suggesting the importance of their functions for ecological fitness. B. gladioli, which also causes BPB of rice, possesses two tofM homologs along with two sets of luxI and luxR homologs. Between the two predicted proteins encoded by the tofM homologs of B. gladioli, one shows the highest level of homology (80% amino acid sequence identity) to TofM, while the other shows only 23.7% identity (Table 3). It is noteworthy that, among the tofM homologs investigated in this study, all of the homologs with greater than 49% identity in deduced amino acid sequence to tofM had the same position and orientation patterns as tofM and rsaM relative to their neighboring luxI and luxR homologs (Table 3 and Figure S3). Regarding the conserved genetic locations and amino acid sequences of encoded proteins, it is very probable that the tofM homologs of other Burkholderia spp., including the select agents, B. mallei and B. pseudomallei, execute similar functions to tofM. Thus, elucidation of the tofM function in the TofI/TofR QS system of B. glumae would provide useful insights into the counter parts of human and animal pathogenic Burkholderia spp.

Conclusively, tofI- and tofR-independent production of toxoflavin in B. glumae was revealed for the first time in this study and tofM was identified as a key genetic component of this newly found pathway for toxoflavin production. tofM alone was also found to contribute to the full virulence of B. glumae 336gr-1. Further studies to determine the regulatory functions of tofM in the expression of tofI and tofR as well as other virulence genes of B. glumae would lead to a better understanding of the global regulatory system that governs the expression of virulence genes in this pathogen and, possibly, other related bacterial species.

Supporting Information

Figure S1 Growth curves of B. glumae strains, 336gr-1 (wild type), LSUPB145 (AtofI), and LSUPB169 (AtofR) grown in LB broth (top left), LB broth amended with 1 μM N-hexanoyl homoserine lactone (C6-HSL) (top right), and LB broth amended with or N-octanoyl homoserine lactone (C8-HSL) (bottom). Bacteria were grown at 37°C in a shaking incubator at ~200 rpm. Similar patterns of data were obtained from three independent experiments.

(TIF)
Figure S2 Toxoflavin production by *B. glumae* strains, LSUPB145 (*tofI*), LSUPB201 (*tofI/*tofR*), LSUPB294 (*tofI/*tofR/*tofM*) and LSUPB139 (*tofI/*tofR/*tofR*) on LB agar plates. Bacteria were inoculated on LB agar plates with the streaking method from fresh colonies of *B. glumae* strains. Toxoflavin production is indicated by the presence of the yellow pigment in the media. Photo was taken after 24 h incubation at 37°C.

Figure S3 A phylogenetic tree of the RsaM homologs from the genome sequences of *Burkholderia* spp. and the relative positions and transcriptional directions of the *rsaM* homologs. The accession number of TofM is indicated with a red box. Red, green, and orange arrows indicate the homologs of *luxC*, *rsaM*, and *luxI*, respectively. Arrow direction indicates the transcriptional direction of depicted genes; arrow size is not proportional to the size of the corresponding genes. The phylogenetic tree was conducted with MEGA5 [27] using the UPGMA method based on the amino acid sequences of the 27 homologs of *B. glumae* indicated with a red box. Red, green, and orange arrows indicate the homologs of *luxC*, *rsaM*, and *luxI*, respectively. Arrow direction indicates the transcriptional direction of depicted genes; arrow size is not proportional to the size of the corresponding genes. The phylogenetic tree was conducted with MEGA5 [27] using the UPGMA method based on the amino acid sequences of the 27 homologs of *B. glumae*. Bootstrap values from 1000 replications were given next to the branches. The numbers indicating the evolutionary distance at the bottom of the tree represent the number of amino acid substitutions per site.

Figure S4 Toxoflavin production of *Burkholderia glumae* mutants and mutants complemented with functional clones of the mutated genes. (A) Toxoflavin production of 336gr-1 (wild type), LSUPB145 (*tofI*), LSUPB169 (*tofR*), LSUPB139 (*tofI/*tofR*) and LSUPB139 with pBBtofIMR. (B and C) Toxoflavin production of 336gr-1 (wild type), LSUPB286 (*tofM*) and LSUPB206 with pBBtofM in LB broth (B) and LB agar (C). Photos were taken at 24 h after incubation at 37°C.

Figure S5 A schematic diagram summarizing the complementation tests conducted in this study. The area deleted in each gene(s) is indicated in a lighter version of the color of the gene. *Toxoflavin production by bacteria inoculated with the streaking method.

Figure S6 AHL production by *B. glumae* strains, 336gr-1 (wild type), LSUPB145 (*tofI*), LSUPB286 (*tofM*), and LSUPB286 complemented with pBBtofM. AHL production by each strain of *B. glumae* is indicated by the production of violacein in the biosensor, *Chromobacterium violaceum* CV026. Photo was taken 48 h after application of *B. glumae* culture extracts on the biosensor and incubation at 30°C.

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Author Contributions

Conceived and designed the experiments: JHH RC. Performed the experiments: RC IKB HSK. Analyzed the data: JHH RC IKB HSK. Contributed reagents/materials/analysis tools: IKB RC. Wrote the paper: JHH RC.

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