Identification of potential genetic components involved in the deviant quorum-sensing signaling pathways of *Burkholderia glumae* through a functional genomics approach

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**Burkholderia glumae** is the chief causal agent for bacterial panicle blight of rice. The acyl-homoserine lactone (AHL)-mediated quorum-sensing (QS) system dependent on a pair of *luxI* and *luxR* homologs, *tofI* and *tofR*, is the primary cell-to-cell signaling mechanism determining the virulence of this bacterium. Production of toxoflavin, a major virulence factor of *B. glumae*, is known to be dependent on the *tofI/tofR QS system. In our previous study, however, it was observed that *B. glumae* mutants defective in *tofI* or *tofR* produced toxoflavin if they grew on the surface of a solid medium, suggesting that alternative signaling pathways independent of *tofI* or *tofR* are activated in that growth condition for the production of toxoflavin. In this study, potential genetic components involved in the *tofI* - and *tofR*-independent signaling pathways for toxoflavin production were sought through screening random mini-Tn5 mutants of *B. glumae* to better understand the intercellular signaling pathways of this pathogen. Fifteen and three genes were initially identified as the potential genetic elements of the *tofI*- and *tofR*-independent pathways, respectively. Especially, the ORF (bglu_2g06320) divergently transcribed from *toxJ*, which encodes an orphan LuxR protein and controls toxoflavin biosynthesis, was newly identified in this study as a gene required for the *tofR*-independent toxoflavin production and named as *toxK*. Among those genes, *flhD*, *dgcB*, and *wzyB* were further studied to validate their functions in the *tofI*-independent toxoflavin production, and similar studies were also conducted with *qsmR* and *toxK* for their functions in the *tofR*-independent toxoflavin production. This work provides a foundation for future comprehensive studies of the intercellular signaling systems of *B. glumae* and other related pathogenic bacteria.

**Keywords:** *Burkholderia glumae*, bacterial panicle blight of rice, bacterial grain rot of rice, quorum-sensing, toxoflavin

**Introduction**

*Burkholderia glumae* causes bacterial panicle blight of rice and produces major virulence factors, such as toxoflavin and lipase, under the control of the quorum sensing (QS) system mediated by the *luxI* homolog, *tofI*, and the *luxR* homolog, *tofR* (Kim et al., 2004, 2007; Devescovi et al., 2007).
Kim et al. (2004) used biosensors and thin layer chromatography to determine the acyl-homoserine lactone ( AHL)-type autoinducers of B. glumae and found that N-octanoyl homoserine lactone (C8-HSL) and N-hexanoyl homoserine lactone (C6-HSL) are produced by the LuxI homolog, TofI. C8-HSL is considered as the functional autoinducer binding to TofR for promoting the virulence-related phenotypes including toxoflavin production and flagellum-mediated motility, while the role of C6-HSL is still vague (Kim et al., 2004, 2007). Several important genes for the virulence of B. glumae regulated by C8-HSL and its cognate receptor TofR include: the tox gene clusters (operons) for toxoflavin biosynthesis ( toxABCDE) and transport ( toxFGHH) (Kim et al., 2004; Shingu and Yoneyama, 2004; Suzuki et al., 2004), lipA encoding the LipA lipase (Devescovi et al., 2007), genes for flagellar biogenesis and qsmR encoding an IcrR-type transcriptional regulator (Kim et al., 2007), and katG encoding a protective catalase (Chun et al., 2009). In addition, genes for the type III secretion system (T3SS) was demonstrated to be a part of the regulon of the tofI/tofR QS system (Kang et al., 2008). As these genes under the control of the tofI/tofR QS is important for the survival, colonization and pathogenesis of B. glumae, it will be beneficial to expand the knowledge upon the intercellular signaling network involving tofI and tofR for gaining a better understanding of the pathogenic behaviors of this pathogen.

In our previous study, a series of deletion mutants of B. glumae for tofI and tofR were generated for comprehensive characterization of the tofI/tofR QS system, using a Louisiana strain of B. glumae, 336gr-1 (Chen et al., 2012). Consistent with the previous studies with mutant derivatives of the B. glumae strain BGR1, ∆tofI or ∆tofR derivatives of B. glumae 336gr-1 did not produce toxoflavin in Luria-Bertani (LB) broth (Chen et al., 2012). However, these mutants produced high levels of toxoflavin when they were grown on solid media, including Luria broth (LB) agar and King’s B (KB) agar (Chen et al., 2012). These results indicate the presence of previously unknown signaling/regulatory pathways for the production of toxoflavin that are activated in certain growth conditions (e.g., solid media) in tofI- and tofR-independent manners (Chen et al., 2012). In this study, ∆ΔtofI and ∆ΔtofR derivatives of B. glumae 336gr-1 were randomly mutagenized with mini-Tn5 Cm and the mutants showing lost ability of toxoflavin production on LB agar were screened in an attempt to identify and characterize the tofI- and tofR-independent signaling/regulatory pathways for toxoflavin production. For more sensitive visual detection of altered toxoflavin production by each transposon mutant, a DNA construct that harbors the promoterless gusA reporter gene fusion to the promoter region of the toxoflavin biosynthesis operon toxABCDE, PtoxABCDE:gusA, was introduced into the ∆ΔtofI and ∆ΔtofR mutants, and the expression of the fused gusA reporter gene in each mutant was monitored based on the blue coloration of each mutant on LB agar containing the substrate of β-glucuronidase, 5-bromo-4-chloro-1H-indol-3-yl β-D-glucopyranosiduronic acid (X-gluc). Mutated genes of the primarily screened mutants, which did not show blue coloration on LB agar with X-gluc, were identified by sequencing the flanking regions of mini-Tn5 Cm inserted in the genome and the functions of selected genes were further studied in terms of their regulatory roles in toxoflavin production and virulence.

Materials and Methods

Growth Conditions of Bacterial Strains

The bacterial strains and plasmid constructs used in this study are listed in Table 1. Media used for routine cultures of bacterial strains were LB broth or LB agar (Sambrook, 2001) with appropriate amendments of antibiotics. Liquid cultures were incubated in a shaking incubator at 200 rpm at 37°C. The antibiotics and their working concentrations used in this study were: ampicillin (Amp), 100 μg/ml; kanamycin (Km), 50 μg/ml; nitrofurantoin (Nit), 100 μg/ml; and gentamycin (Gm), 20 μg/ml. The substrate of β-glucuronidase, X-gluc, was applied at a working concentration of 2 mM.

Recombinant DNA Techniques

General DNA manipulations were conducted following standard methods (Sambrook, 2001). A StrataClone™ PCR cloning kit (Agilent Technologies, Santa Clara, CA, USA) was used for cloning PCR products into a pSC-A-amp/kan vector. Cloned PCR products in pSC-A-amp/kan were sequenced for confirmation at GeneLab in the LSU School of Veterinary Medicine or at Macrogen USA (http://www.macrogenusa.com) using M13 forward and reverse primers. A GenePulser unit (BioRad Laboratories, Hercules, CA, USA) was used to perform electroporation for transforming E. coli competent cells under 1.5 kV, with 1 μl of ligated DNA and 25 μl of competent cells. Triparental mating was used for the transformation of B. glumae with DNA constructs (Figurski and Helinski, 1979). Digested DNA fragments were extracted from the agarose gel using GenElute™ Gel extraction kits (Sigma-Aldrich, St. Louis, MO, USA). The concentrations of the purified DNA and RNA were measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All the restriction enzymes used in this study were purchased from New England Biolabs (Beverly, MA, USA). The genomic library used in this study was constructed previously in our laboratory (Karki et al., 2012a).

Construction of pKGpToxA-GUS

To generate the DNA construct for a gusA fusion to the promoter region of toxABCDE (PtoxABCDE:gusA), the DNA fragment containing 562 bp upstream and 108 bp continuous coding sequences of toxA was amplified with the primer set, GUS/PtoxA. The resultant PCR product was inactivated in pSC-A-amp/kan, generating pSC-A-pToxA. The cloned region was then introduced into pBB2GUS, a broad host vector containing a promoterless gusA, using the unique HindIII site to obtain pBB2GUS-pToxA. The region containing the PtoxABCDE::gusA fusion was cut with KpnI and SpeI and ligated to the suicide vector, pKNOCK-Gm (Alexeyev, 1999), to generate pKGpToxA-GUS. This DNA construct was then introduced into the ΔΔtofI strain, LSUPB145, and the ΔΔtofR strain, LSUPB289, through tri-parental mating, generating LSUPB178 and LSUPB324, respectively.

Random Mutation of B. glumae and Screening of Random B. glumae Mutants

Mini-Tn5 Cm, a mini-Tn5 transposon carrying a Cm resistant gene (De Lorenzo et al., 1990), was introduced to LSUPB178
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or Plasmid | Description | References |
|-------------------|-------------|-----------|
| **Escherichia coli** |  |  |
| DH10B | F^- araD139 Δ[ara, leu]7697 ΔlacX74 galU galK rpsL deoR o80dIacZΔM15 endA1 nupG recA1 mcrA Δ[mrn hsdRMS mcrBC] | Grant et al., 1990 |
| DH5α | F^- Δ[Δ1]hisD17 (g′, m') supE44 thi-1 rgyA96 relA1 deoR Δ[lacZYA-argF]-U169 o80dIacZΔM15 | Grant et al., 1990 |
| S17-1 λpir | recA thi pro hsdR Δ[es- modI]RP4::2-Tc::Mu-Km::Tn7] λ pir phage lysogen, SmR'/Tp' | Simon et al., 1983 |
| **Burkholderia glumae** |  |  |
| 336gr-1 | Wild type strain and the causative isolate of bacterial panicle blight of rice in Crowley, LA | This study |
| LSUPB22 | spontaneous mutant of 336gr-1 | This study |
| LSUPB145 | A ΔtofM derivative of 336gr-1 | Chen et al., 2012 |
| LSUPB169 | A ΔtofR derivative of 336gr-1 | Chen et al., 2012 |
| LSUPB139 | A Δtof-tofR derivative of 336gr-1 | Chen et al., 2012 |
| LSUPB286 | A ΔtofM derivative of 336gr-1 | Chen et al., 2012 |
| LSUPB172 | A derivative of 336gr-1 carrying pKGpToxA-GUS | This study |
| LSUPB178 | A derivative of ΔtofI carrying pKGpToxA-GUS | This study |
| LSUPB324 | A derivative of ΔtofR carrying pKGpToxA-GUS | This study |
| LSUPB503 | A thd^- derivative of the ΔtofI strain, LSUPB145 | This study |
| LSUPB273 | A qsmR^- derivative of the wild type strain, 336gr-1 | This study |
| LSUPB275 | A qsmR^- derivative of the ΔtofI strain, LSUPB145 | This study |
| LSUPB277 | A qsmR^- derivative of the ΔtofR strain, LSUPB169 | This study |
| LSUPB445 | A toxK^- derivative of the ΔtofR strain, LSUPB169 | This study |
| LSUPB460 | A dgcB^- derivative of the wild type strain, 336gr-1 | This study |
| LSUPB462 | A dgcB^- derivative of the ΔtofI strain, LSUPB145 | This study |
| LSUPB464 | A dgcB^- derivative of the ΔtofM strain, LSUPB286 | This study |
| LSUPB615 | A wzyB^- derivative of the ΔtofI strain, LSUPB145 | This study |
| LSUPB616 | A wzyB^- derivative of the ΔtofR strain, LSUPB169 | This study |
| **Chromobacterium violaceum** |  |  |
| Chromobacterium violaceum CV026 | A biosensor that can detect AHL molecules | McClean et al., 1997 |
| **Plasmid** |  |  |
| pSC-A-amp/kan | A blunt PCR cloning vector; f1 ori, pUC ori, lacZ', AmpR | Stratagene |
| pRK2013::Trn7 | A helper plasmid; CoIE1 ori | Ditta et al., 1980 |
| mini-Tn5 Cm | A derivative of mini-Tn5 transposon, R6K ori, RP4 oriT, CmR | De Lorenzo et al., 1990 |
| pKNOCK-Km | A suicide vector; R6K ori, RP4 oriT, KmR | Alexeyev, 1999 |
| pKNOCK-Gm | A suicide vector; R6K ori, RP4 oriT, GmR | Alexeyev, 1999 |
| pBBR1MCS-2 | A broad host range cloning vector, RK2 ori, lacZa, KmR | Kovach et al., 1995 |
| pBB2GUS | a derivative of pBBR1MCS-2 containing a promoterless gusA | This study |
| pSC-A-pToxA | A PCR clone of 682-bp upstream promoter and partial coding region of toxA in pSC-A-amp/kan, AmpR, KmR | This study |
| pBB2GUS-pToxA | A subclone of pSC-A-pToxA for the promoter and partial coding region of toxA in pBB2GUS at HindIII site | This study |
| pKGpToxA-GUS | A subclone of pBB2GUS-pToxA for the promoter and partial coding region of toxA gene and gusA gene in pKNOCK-Gm at KpnI and Spel sites | This study |
| pSC-qsmR | A PCR clone of 355-bp internal region of qsmR gene in pSC-A-amp/kan, AmpR, KmR | This study |
| pSC-thd | A PCR clone of 304-bp internal region of thdD gene in pSC-A-amp/kan, AmpR, KmR | This study |
| pSC-toxA | A PCR clone of 402-bp internal region of toxK gene in pSC-A-amp/kan, AmpR, KmR | This study |
| pSC-dgcB | A PCR clone of 376-bp internal region of dgcB gene in pSC-A-amp/kan, AmpR, KmR | This study |
| pSC-wzyB | A PCR clone of 370-bp internal region of wzyB gene in pSC-A-amp/kan, AmpR, KmR | This study |
| pkKmqsR | A subclone of pSC-qsmR for the internal region of qsmR gene in pKNOCK-Km at KpnI and SacI sites, KmR | This study |
| pkKmtthd | A subclone of pSC-thdD for the internal region of thdD gene in pKNOCK-Km at EcoRI site, KmR | This study |
| pkKmttoxK | A subclone of pSC-toxA for the internal region of toxK gene in pKNOCK-Km at EcoRI site, KmR | This study |
| pkGndaB | A subclone of pSC-dgcB for the internal region of dgcB gene in pKNOCK-Km at Spel and HindIII sites, KmR | This study |
| pkGnmwzyB | A subclone of pSC-wzyB for the internal region of wzyB gene in pKNOCK-Gm at Spel and KpnI sites, KmR | This study |
(a ΔtofI derivative carrying the P_{toxABCDE}::gusA fusion in the genome) and LSUPB324 (a ΔtofR derivative carrying the P_{toxABCDE}::gusA fusion in the genome) by tri-parental mating for random mutagenesis. After the tri-parental mating, mini-Tn5 Cm mutants that did not show blue coloration (indicating no β-glucuronidase gene activity) on LB/Gm/Cm/Nfr/X−gluc plates after 2 days of incubation at 37°C were collected for identifying the genes disrupted by mini-Tn5 Cm.

### Identification of Genes Disrupted by Mini-Tn5 Cm

The mutated genes of the screened mutants were identified following a previously developed method (Kwon and Ricke, 2000) with some modifications. Briefly, genomic DNA of mutant strains were extracted using a GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). Genomic DNA (ca. 4 mg) was digested with two restriction enzymes, PstI and NalIII, and the digested DNA fragments were ligated to the Y-shaped linker. Then, Y Linker Primer, CTGCTCGAAGTCAAGGAG (specific to Y linker sequence), and Tn5 primer, GGCAGATCGATCAAGAG (specific to transposon), were used to amplify the DNA sequences flanking where the transposon was inserted. The amplified PCR products were sequenced and the sequence data were BLAST-searched against the National Center for Biotechnology Information (NCBI) database for identification of the sequenced region.

### Directional Mutation for Validation of Gene Functions

A 300—400 bp internal DNA sequence of a gene of interest was amplified using a primer set indicated in Table 2 and cloned into a pKNOCK suicide vector, pKNOCK-Km or pKNOCK-Gm (Alexeyev, 1999). Specifically, PCR products for 355 bp of qsmR, 304 bp of flhD, 402 bp of toxK, 376 bp of dgcB and 370 bp of wzyB were cloned into PSC-A-amp/kan to generate PSC-qsmR, PSC-flhD, PSC-toxK, PSC-dgcB and PSC-wzyB, respectively. KpnI and SacII sites were used to transfer the qsmR region in PSC-qsmR to pKNOCK-Km to generate pKKmqsmR. EcoRI site was used to move the flhD and toxK regions in PSC-flhD and PSC-toxK to PSC-dgcB and PSC-wzyB, respectively. SpeI and HindIII were used to transfer the dgcB region from pSC-dgcB to pKNOCK-Gm, generating pKGmdgcB. SpeI and KpnI were used to transfer the wzyB region from pSC-wzyB to pKNOCK-Gm, generating pKgmwzyB. Each final DNA construct in a pKNOCK suicide vector was introduced into B. glumae strains by triparental mating. Diagnostic PCR was conducted to confirm if the internal sequence of each gene was successfully integrated to the bacterial genome through homologous recombination. Primer pairs listed in Table 2, FLHD-C1/FLHD-C2, toxK-C1/toxK-C2, QsmR-C1/QsmR-C2, DGC-B1/DGC-B2, and Oap-C1/Oap-C2, were used for the diagnostic PCRs to confirm...
the targeted mutations of flhD, toxK, qsmR, dgcB, and wzyB, respectively.

Quantification of Toxoflavin Production by B. glumae Strains Grown on LB Agar

Toxoflavin produced by B. glumae strains grown on LB agar was quantified following a previous method (Chen et al., 2012) with miscellaneous modifications. Briefly, overnight bacterial cultures were concentrated to OD600 = 100 (~10^11 cfu/ml) and one loopful of each inoculum was streaked on the entire area of a LB agar plate. After 24 h incubation at 37°C, the bacterial culture was scraped off from the surface of the LB agar. Five grams of the LB agar where bacteria were grown on was then cut into small pieces with a razor blade. The chopped agar pieces were mixed with 5 ml chloroform and left to sit at room temperature for 30 min. The chloroform fraction was then transferred to a new tube and air-dried under a fume hood. The remaining materials in the dried tube were dissolved in 1 ml of 80% methanol. The OD_{393nm} values were measured to determine the quantity of toxoflavin, using a spectrophotometer. The sample from uncultured LB agar was used to set the zero of OD_{393nm}.

Virulence Assay with Onion Bulb Scales

Virulence tests for the mutant strains of B. glumae were conducted using onion bulbs as a surrogate host, following a previously described method (Karki et al., 2012b).

Results

Fifteen Protein-Coding Genes Were Identified as Potential Genetic Components for the TofI-Independent Production of Toxoflavin

Both LSUPB178 (a ΔtofI derivative carrying the P_{toxA::gusA} fusion in the genome) and LSUPB324 (a ΔtofR derivative carrying the P_{toxA::gusA} fusion in the genome) showed blue coloration on LB agar containing X-glu, indicating that the introduced gusA fusion to the toxA promoter is functional (data not shown). After the random mutagenesis of LSUPB178 with mini-Tn5 Cm, the mutant colonies that did not exhibit blue color were picked from the selection plates (LB agar amended with Gm, Cm, Nit, and X-glu). It was considered that mini-Tn5 Cm is inserted in a potential genetic component of the tofI-independent toxoflavin production pathway in the mutants screened. Among the 4400 random mini-Tn5 Cm derivatives of LSUPB178 observed, 16 mutant derivatives were initially screened and their mutated genes were identified through BLAST searches of the flanking sequences (Table 3). All the 16 mutant strains screened were confirmed to be toxoflavin-deficient on LB agar at 37°C (data not shown). Fifteen out of the 16 mutants screened turned out to have mini-Tn5 Cm insertion in protein-coding regions and one mutant had the insertion in the middle of a 23S ribosomal RNA sequence. The 15 protein-coding genes identified include toxR and toxA, which are known to be required for toxoflavin production (Herrmann and Weaver, 1999), indicating the validity of this experiment (Table 3). Among the 15 genes identified, three genes (flhD encoding a transcriptional activator for flagellar biogenesis, dgcB encoding a putative diacylglycl late cyclase, and wzyB encoding a putative O-antigen polymerase family protein) were selected for the extended functional studies described below.

TABLE 3 | The list of potential genes contributing to the tofI-independent toxoflavin production.

| Name of random mutants | Locus of inserted genes | Function of disrupted genes |
|------------------------|------------------------|----------------------------|
| LSUPB186               | bglu_1g10100           | Succinylornithine transaminase |
| LSUPB187               | bglu_2g10840*          | putative LuxS domain-containing protein |
| LSUPB182               | bglu_1g02180           | Diguanylate cyclase |
| LSUPB183               | bglu_1g01780*          | Flagellar transcriptional activator FlhD |
| LSUPB184               | bglu_2g07160*          | Catecho1, 1,2-dioxygenase |
| LSUPB185               | bglu_1g00380           | General secretory pathway protein D |
| LSUPB188               | bglu_2g20200*          | Hypothetical protein |
| LSUPB189               | bglu_1g07800           | Hypothetical protein |
| LSUPB192               | bglu_2g06390           | LysR family transcriptional regulator (toxR) |
| LSUPB193               | bglu_2g06400*          | putative ubiquinone/maeauquinone biosynthesis methyltransferase (toxA) |
| LSUPB194               | bglu_2g18120           | Amylo-alpha-1,6-glucosidase family protein |
| LSUPB209               | bglu_1g30370           | Flagellar hook-associated protein FgK |
| LSUPB210               |                        | rRNA-23S ribosomal RNA |
| LSUPB211               | bglu_1g29900           | O-antigen polymerase family protein |
| LSUPB212               | bglu_1g00440           | Glutamate-cysteine ligase |
| LSUPB213               | bglu_1g05190           | Short chain dehydrogenase |

*Mini-Tn5Cm is inserted in the promoter region.

Three Protein-Coding Genes Were Identified as Potential Genetic Components for the tofR-Independent Production of Toxoflavin

Similar to LSUPB178, LSUPB324 (ΔtofR: P_{toxA::gusA}) exhibited blue color on LB agar X-gluc (data not shown). Three mini-Tn5 Cm derivatives of LSUPB324 that did not produce blue color were characterized for the mutated genes (Table 4), and loss of the function in toxoflavin production was observed with all the three mutants (data not shown). Particularly, one toxoflavin-deficient mini-Tn5 Cm mutant, LSUPB191, turned out to be disrupted in the ORF, bglu_2g06320 (Table 4). This ORF is located upstream of and divergently transcribed from toxJ, a known regulatory gene for toxoflavin biosynthesis encoding an orphan LuxR protein. Due to its apparent function in toxoflavin production, the ORF was newly named as toxK in this study. Among the three identified genes, qsmR and toxK were chosen for the further functional studies described below.

Targeted Mutation of flhD, dgcB, wzyB, qsmR, or toxK Resulted in Impaired Toxoflavin Production

To confirm the initially observed functions of flhD, dgcB, and wzyB in tofI-independent toxoflavin production, independent mutants of these genes were generated via homologous recombination. For this, internal regions of flhD, dgcB, and wzyB were cloned in a suicide vector (pKNOCK-Km or pKNOCK-Gm) and introduced into the ΔtofI background, LSUPB145, generating the corresponding mutants, LSUPB503, LSUPB462, and LSUPB515,
respectively (see the Materials and Methods section for detail). The same strategy was applied for the targeted mutation of \textit{qsmR} and \textit{tofK} in the \textit{\Delta tofR} background, LSUPB169, generating the corresponding mutants LSUPB277 and LSUPB445, respectively. These independent mutants of the five genes were then examined for their phenotypes in toxoflavin production in comparison with their parental strains.

\textit{flhD}

Compared to LSUPB145, its \textit{flhD}\textsuperscript{−} derivative LSUPB503 showed a substantial reduction in toxoflavin production (Figure 1). There was little difference between LSUPB145 and LSUPB503 during the first 24 h of incubation period, but during the additional 24 h incubation period, reduction of toxoflavin amount was observed with LSUPB503, while more than 5 times increase of toxoflavin was observed with LSUPB145 during the same incubation period (Figure 1). Null mutation of \textit{flhC}, which is located downstream of \textit{flhD}, in the \textit{\Delta tofI} background through homologous recombination of an internal coding region also caused loss of toxoflavin production (data not shown).

\textbf{TABLE 4 | The list of tofR-independent genes contributing to tofR-independent toxoflavin production.}

| Name of random mutants | Locus of inserted genes | Function of disrupted genes |
|-------------------------|-------------------------|-----------------------------|
| LSUPB190                | bglu_1g10250            | TofR family regulatory protein gene (qsmR) |
| LSUPB191                | bglu_2g06320            | Hypothetical protein in the upstream of toxJ encoding an orphan LuxR family transcriptional regulator (named as toxK in this study) |
| LSUPB195                | bglu_2g22590            | Putative PAS/PAC sensor protein |

\textbf{FIGURE 1 | Toxoflavin production of LSUPB503 (\textit{flhD}\textsuperscript{−}/\textit{\Delta tofI}) compared to its parental strain, LSUPB145 (\textit{\Delta tofI}), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD\textsubscript{590}/g LB agar values.}

\textit{dgCB}

Both of the two independent \textit{dgCB} null mutants, LSUPB462 (a \textit{dgCB::pKGMdgCB} derivative of the \textit{\Delta tofI} strain LSUPB145) and LSUPB182 (a \textit{dgCB::mini-Tn5 Cm} derivative of LSUPB145), were toxoflavin-deficient on LB agar medium in common (data not shown). This indicates the important role of the DgcB diguanylate cyclase in the \textit{tofI}-independent pathway for toxoflavin production. To know if \textit{dgCB} is also critical for toxoflavin production when the \textit{tofI/tofR} QS system is intact, LSUPB460 (a \textit{dgCB}\textsuperscript{−} derivative of the wild type strain 336gr-1) was generated and examined for toxoflavin production. As shown in Figure 2, LSUPB460 produced a similar level of toxoflavin production compared to 336gr-1. This suggests that \textit{dgCB} plays a positive role in toxoflavin production but this function is dispensable in the presence of the intact \textit{tofI/tofR} QS system. To explore the relationship between \textit{dgCB} and other QS elements \textit{tofR} and \textit{tofM} (Chen et al., 2012), \textit{pKGMdgCB} was introduced into LSUPB169 (a \textit{\Delta tofR} mutant) and LSUPB286 (a \textit{\Delta tofM} mutant) for generating \textit{dgCB}\textsuperscript{−}-derivatives of these QS mutant strains. But \textit{dgCB}\textsuperscript{−}-derivatives could be obtained from only LSUPB286 but not LSUPB169, and the \textit{dgCB}\textsuperscript{−}/\textit{\Delta tofM} strain, LSUPB464, also showed a toxoflavin-deficient phenotype like its parent, LSUPB286 (Figure 2).

\textit{wzyB}

LSUPB15, the \textit{wzyB} disruptive mutant in LSUPB145 background grew very slowly on both LB agar (data not shown) and LB broth (Figure 3A). Because of the extremely slow growth of the bacterial cells, it was difficult to conduct reliable toxoflavin quantification for LSUPB15. To determine if \textit{wzyB} is also required for the \textit{tofR}-independent toxoflavin production, a disruptive mutant in LSUPB169 background, LSUPB156, was generated and tested for its ability to produce toxoflavin. LSUPB156 showed normal cell growth unlike LSUPB15 (Figure 3A) and a toxoflavin-deficient phenotype (Figure 3B), indicating that \textit{wzyB} is required for both \textit{tofI} and \textit{tofR}-independent toxoflavin production. Generation of a \textit{wzyB} null mutant derivative of 336gr-1 to determine the role of this gene in the wild type background was not successful.

\textit{qsmR}

LSUPB277, \textit{qsmR} mutated in LSUPB169 background, had the same toxoflavin loss phenotype as the random mutant LSUPB190, which confirmed the important role of \textit{qsmR} in \textit{tofR}-independent toxoflavin production (Figure 4). To determine the role of \textit{qsmR} in toxoflavin production in the presence and absence of the intact QS system, LSUPB273 (a \textit{qsmR} mutant in the wild type background 336gr-1) and LSUPB275 (a \textit{qsmR} mutant in the \textit{\Delta tofI} background LSUPB145) were also generated and tested for their phenotypes in toxoflavin production. As shown in Figure 4, all the \textit{qsmR} knock out mutants tested were deficient in toxoflavin production regardless of the genetic background of their parental strains, indicating the essential role of \textit{qsmR} in the production of toxoflavin.
FIGURE 2 | Toxoflavin production of the dgcB mutants, LSUPB460 (dgcB−), LSUPB462 (dgcB−/ΔtofI) and LSUPB464 (dgcB−/ΔtofM), compared to their parental strains, 336gr-1 (wild type), LSUPB145 (ΔtofI) and LSUPB286 (ΔtofM), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD393/g LB agar values.

FIGURE 3 | Bacterial growth and toxoflavin production of wzyB mutants. (A) Bacterial growth in LB broth at 37°C: LSUPB145 (ΔtofI), LSUPB169 (ΔtofR), LSUPB503 (ΔlilD− in ΔtofI), LSUPB445 (toxK− in ΔtofI), LSUPB273 (qsmR−), LSUPB275 (qsmR− in ΔtofI), LSUPB277 (qsmR− in ΔtofR), LSUPB460 (dgcB−), LSUPB462 (dgcB− in ΔtofI), LSUPB464 (dgcB− in ΔtofM), and LSUPB516 (wzyB− in ΔtofR). Photo was taken 48 h after inoculation. (B) Toxoflavin production of the wzyB mutant, LSUPB516 (wzyB−/ΔtofR), compared to its parental strains, LSUPB169 (ΔtofR), grown on LB agar after 24 and 48 h of incubation at 37°C. The amounts of toxoflavin are indicated with the OD393/g LB agar values.

**toxK**
As shown in Figure 5, toxoflavin production was almost abolished in the toxK mutant in LSUPB169 background, LSUPB445, indicating that this putative gene in front of toxI encoding an orphan LuxR protein is essential for toxoflavin production of *B. glumae* in the absence of tofR. The function of toxK in the wild type and ΔtofI backgrounds has not been determined due to the failure of generation of toxK− and toxK−/ΔtofI strains through homologous recombination.

**Onion Maceration Caused by Mutants**
The virulence functions of the five selected genes were determined with the virulence assay system using onion bulb scales as a surrogate host (Karki et al., 2012a,b). The strains included in the virulence assay were; 336gr-1 (wild type), LSUPB145 (ΔtofI), LSUPB169 (ΔtofR), LSUPB286 (ΔtofM), LSUPB503 (ΔlilD− in ΔtofI), LSUPB445 (toxK− in ΔtofI), LSUPB273 (qsmR−), LSUPB275 (qsmR− in ΔtofI), LSUPB277 (qsmR− in ΔtofR), LSUPB460 (dgcB−), LSUPB462 (dgcB− in ΔtofI), LSUPB464 (dgcB− in ΔtofM), and LSUPB516 (wzyB− in ΔtofR). As shown in Figure 6, all the mutants tested exhibited reduced virulence compared to their parental strains even though none of them were completely avirulent, indicating the positive roles of the five genes in bacterial pathogenesis.

**Discussion**

**Genome-Wide Screening by Random Transposon Mutagenesis Revealed Novel Regulators of Toxoflavin in *B. glumae***
The toxA promoter and gusA transcriptional fusion was introduced to ΔtofI and ΔtofR mutants and random mutagenesis with mini-Tn5 Cm was implemented to search for the genes that could affect the expression of the toxABCDE operon, thus the toxoflavin biosynthesis, in tofI- and tofR-independent ways. Through this approach, fifteen and three protein-coding genes were identified as potential regulators for the tofI- and tofR-independent toxoflavin biosynthesis of *B. glumae* 336gr-1, respectively. Identification of toxA and toxR, which are known genes required for toxoflavin biosynthesis, verifies the reliability of this experiment. The reason for the abolishment of the P_toxA:gusA expression by disruption of toxA may be due to the fact that ToxR, which is an essential regulatory factor for toxoflavin biosynthesis, requires a
residual amount of toxoflavin as a co-inducer for its functionality (Kim, 2004). Among the potential genetic elements for tofI- or tofR-independent toxoflavin production identified from screening of random mini-Tn5 Cm mutants, five genes (flhD encoding a flagella transcriptional activator, dgcB encoding a diguanylate cyclase, wzyB encoding an O-antigen polymerase family protein, qsmR encoding an IclR-type transcriptional regulator, and toxK encoding a hypothetical protein and divergently transcribed from toxJ encoding an orphan LuxR homolog essential for toxoflavin production) were further characterized in this study for the ir functions in toxoflavin production in the presence or absence of the intact TofI/TofR QS system.

Based on the annotated genome B. glumae BGR1 (Lim et al., 2009), it is unlikely that possible polar effects from the transpo-son or directional mutations can cause misinterpretation of the observed mutant phenotypes for the five genes investigated in this study. flhD (bglu_1g01780) and flhC (bglu_1g01790) comprise an operon, but their products are known to work together as one functional unit (Kim et al., 2007). In this study, both flhD− and flhC− mutants showed the toxoflavin-deficient phenotype in the ΔtofI background. In case of the other genes, dgcB (bglu_1g02180), qsmR (bglu_1g10250), and toxK (bglu_2g06320) are apparently monocistronic, while wzyB (bglu_1g29900) is located at the end of a putative operon structure containing three genes. Thus, polarity may not be a major concern with the five genes investigated in this study.

**FlhD is Required for the Toxoflavin Production by B. glumae 336gr-1 in the ΔtofI Background**

The flhDC operon encoding the FlhDC complex was characterized as a flagellar transcriptional activator, which was controlled by QS in B. glumae BGR1 (Kim et al., 2007). It was reported that a flhD deficient mutant of BGR1 still produced toxoflavin but lost pathogenicity as well as flagellum-mediated motility (Kim et al., 2007). In this study with another strain of B. glumae, 336gr-1, it was observed that flhD as well as flhC were required for the production of toxoflavin in the absence of tofI. At this point, it has not been determined if flhD− or flhC− derivatives of 336gr-1 still produce toxoflavin like BGR1. Mutation of these genes in the wild type as well as in the ΔtofR backgrounds is currently under way. Due to the significant difference between BGR1 and 336gr-1 in the QS-mediated regulation of toxoflavin, it is possible that flhDC is not involved in toxoflavin production in BGR1. Nevertheless, this study indicates that flhDC has a regulatory function in toxoflavin production in addition to flagellum-mediated motilities in at least some strains of B. glumae.

**dgcB is Required for Toxoflavin Production in the Absence tofI but Not in the Wild Type Background Having the Intact tofI/tofR QS system**

The results in this study indicate that dgcB is required for toxoflavin production in the absence of tofI but not in the presence
of the intact TofI/TofR QS system. dgcB codes for a diguanylate cyclase, which synthesizes cyclic di-guanosine monophosphate (c-di-GMP) from two guanosine triphosphate (GTP) molecules. c-di-GMP is a small diffusible signal molecule that influences a wide range of cellular functions including bacterial virulence (Romling, 2012). The phenotype of the dgcB mutant, LSUPB462, observed in this study strongly implies that the c-di-GMP signaling plays a pivotal role in the regulation of virulence factors in B. glumae. Involvement of c-di-GMP signaling pathways for the pathogenesis by plant pathogenic bacteria has been studied mainly with Xanthomonas spp. Ham (2013). Regulatory functions of c-di-GMP for the expression of EPS and biofilm were recently reported with B. cenocepacia (Fazli et al., 2011, 2013; Deng et al., 2012). Even though this study adds to our knowledge about the function of c-di-GMP signaling in plant pathogenic bacteria, more studies including identification and functional characterization of other c-di-GMP signaling components, such as phosphodiesterases and c-di-GMP-binding protein, should be followed to clearly understand the role of this signaling system for the bacterial pathogenesis by B. glumae.

**wzyB Encoding an O-Antigen Polymerase May Play an Important Role in the tofI-Independent Intercellular Signaling for Toxoflavin Production**

wzyB was initially identified as a genetic element for the tofI-independent toxoflavin production through screening of random mini-Tn5::Cm mutant derivatives of the ΔtofI strain, LSUPB145. LSUPB515, an independent wzyB mutant derivative of LSUPB145 generated through homologous recombination, grew slowly due to an unknown reason and produced no observable toxoflavin. LSUPB516, a wzyB mutant derivative of the ΔtofR strain LSUPB169 through homologous recombination, also lost the ability to produce toxoflavin on a solid medium. These observations indicate that wzyB is required for both tofI- and tofR-independent toxoflavin production. However, it remains to be determined whether or not wzyB is also required for the toxoflavin production in the presence of the intact TofI/TofR QS system because wzyB mutation in the wild type background has been failed due to an unknown reason.

O-antigen is one of the three major components (the other two are lipid A and a core oligosaccharide) of lipopolysaccharides (LPS) in the outer membrane of Gram negative bacteria; and is structurally very diverse and highly immunogenic (Raetz and Whitfield, 2002; Stone et al., 2012). Study of O-antigen and its synthesis pathway can provide important antibiotic targets for potential clinical use. O-antigen polymerase family proteins (encoded by wzy homologs) are responsible for polymerizing the O-antigen units to O-antigen chain (Daniels et al., 1998; Chin et al., 2010). It is also well known that LPS is a common virulence determinant (Thomsen et al., 2003; Ellis and Kuehn, 2010). As an important synthesis enzyme for O-antigen and LPS, O-antigen polymerase should be important for maintaining the virulence of pathogenic bacteria. Even though not much study has been focused on wzy homologs, it was recently reported that non-polar mutation of the wzy gene in Salmonella enterica serovar Typhimurium caused attenuated virulence in mice (Kong et al., 2011). It would be an appealing hypothesis that the outer membrane-bound O-antigen produced by wzy genes mediates signal transduction dependent on “physical contact” of bacterial cells populated on a solid surface.

Furthermore, it would be worthy to make more investigation on the relationship between motility genes, especially flhDC, and wzyB as well as other LPS synthesis genes. There have been reports about how the subunits of and enzymes involved in assembling LPS influence the motility function of bacteria. The mutation of an O-antigen gene in Salmonella enterica serovar Typhimurium resulted in defective bacterial swimming and, in the same study, it was also suggested that the role of O-antigen is to improve the wettability of the bacterial colony (Toguchi et al., 2000). The mutation of wzxE gene, which is responsible for transporting O-antigen units across the inner membrane to the LPS assembling site (Chin et al., 2010), caused E. coli to lose both swimming and swarming motility (Giris et al., 2007). The mutation of waaL, an O-antigen ligase gene, in Vibrio fischeri resulted in loss of motility and ability to survive with the wild type in the co-colony assay (Buttner, 2012). In another study, mutation of waaL blocked the expression of flhDC genes and aborted the swarming motility of human pathogen Proteus mirabilis in soft
agar, but overexpression of flhDC genes in trans overcame this defect (Morgenstein et al., 2010). Therefore, motility assays are under way in our laboratory to determine the functional relationship between wzyB and the QS-regulated motility system in *B. glumae*.

**qsmR is an Essential Regulatory Gene for the Toxoflavin Production of *B. glumae* 336gr-1**

In this study, null mutation of qsmR resulted in lost function of toxoflavin production in the wild type (336gr-1, ΔtofI (LSUPB145) and ΔtofR (LSUPB169) backgrounds, indicating that qsmR is another essential regulatory factor for toxoflavin production in *B. glumae* 336gr-1. QsmR was previously reported as a positive regulator for flhDC, which was activated by C8-HSL and ToFR complex in another strain of *B. glumae*, BGR1 (Kim et al., 2007). In the same study, disruption of qsmR resulted in loss of motility and pathogenicity in rice, but overexpression of qsmR showed decreased production and degradation of toxoflavin in the liquid medium condition from 12 h of incubation (Kim et al., 2007). In this study with *B. glumae* 336gr-1, the qsmR mutant (LSUPB273) did not produce observable toxoflavin on LB agar and still retained partial virulence on onion, which was somewhat different from the results of Kim et al. with *B. glumae* BGR1. These results from this and previous studies suggest that toxoflavin biosynthesis is regulated by qsmR more tightly in 336gr-1 than in BGR1. qsmR was also shown to regulate the katG catalase gene, which is required for the full virulence of *B. glumae* BGR1 (Chun et al., 2009).

Additionally, recent studies with *B. glumae* BGR1 demonstrated that qsmR is a key player for both bacterial pathogenesis and survival in stressful conditions. From a proteomics search, the type II secretion system of *B. glumae* BGR1 encoded by gsp genes was found to be regulated by QS, and QsmR was shown to be a major positive regulator for gsp genes (Goo et al., 2010). In a study about how QS systems help representative *Burkholderia* species survive stationary phases, the sub-QS regulator QsmR was determined to be critical for activating the production of oxalate and maintaining normal pH levels of bacterial culture media (Goo et al., 2012). Without functional QsmR, there was a survival defect in *B. glumae* and *B. thailandensis* populations, which correlated with the high pH and lack of oxalate (Goo et al., 2012). Taken this and the previous studies on qsmR together, it is thought that qsmR plays an important role in general in the expression of various virulence factors in *B. glumae* despite some functional variations among different strains.

**Discovery of the toxK Gene Adds More Complexity to the Known Regulatory Cascade for Toxoflavin Biosynthesis Involving the Orphan LuxR Protein, ToxJ**

The ORF (gene ID of the reference sequence: bglu_2g06320) located upstream of and divergently transcribed from tofI, the QS-dependent toxoflavin regulator encoding an orphan LuxR protein, was identified as a new genetic element required for the tofR-independent toxoflavin production of *B. glumae* 336gr-1 and named as toxK. There is one lux box like sequence (tof box) between tofI and toxK, and it was previously proven that ToFR and C8-HSL complex can bind on that site and activate the expression of tofI (Kim et al., 2004). It may be the case that the tof box between tofI and toxK promotes the expression of both genes, even though this notion should be proved experimentally. The deduced protein encoded by toxK contains 144 residues with 15.5 kDa of estimated molecular mass without an obvious homolog. Instead, the deduced protein (ID: ACR31068) has a conserved domain found in heterogeneous ribonucleoproteins (hnRNPs), implying that binding to target RNAs is its major functional mechanism.

It is interesting that the orphan lux homolog, tofI, is next to an RNA-binding protein-coding gene instead of a luxI homolog. Regarding its location, size and predicted RNA-binding activity, toxK may be considered as a “chaperone” that modulates the expression and function of tofI. More information about the function of toxK will be obtained when toxK mutants in the wild type and ΔtofR backgrounds are available and more phenotypic assays with toxK mutants in various genetic backgrounds are performed. In addition, future molecular studies to determine if mRNA of toxK is a target of ToxR and how ToxR affect the expression of toxK as well as other potential target genes will provide valuable insights into the functional basis of ToxJ and, further, into a type of functional mechanism adopted by other LuxR solos.

In conclusion, potential genetic components involved in the deviant quorum-sensing signaling pathways of *B. glumae* for the production of the major virulence factor, toxoflavin, were identified in this study. In particular, the genes known for other functions, flhDC, dgcB, wzyB, and qsmR, as well as a previously unknown gene, toxK, were newly found to function in the tofI- and tofR-independent toxofalvin production via directional mutation of each gene. The information obtained from this study provides not only great insights into the complex cell-to-cell signaling network of this bacterium but a good foundation of future studies for comprehensive functional analyses of the individual genes identified in this study.

**Acknowledgments**

This study was supported by the USDA NIFA ( Hatch Project #: LAB93918 and LAB94203), the Louisiana State University Agricultural Center, and the Louisiana Board of Regents (the Research and Development Program: LEQSF(2008-11)-RD-A-02). RC was supported by the Economic Development Graduate Assistantship and the Flagship Graduate Assistantship from Louisiana State University Graduate School.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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