Differential regulation of toxoflavin production and its role in the enhanced virulence of *Burkholderia gladioli*

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SUMMARY

Burkholderia gladioli is a causal agent of bacterial panicle blight and sheath/grain browning in rice in many countries. Many strains produce the yellow pigment toxoflavin, which is highly toxic to plants, fungi, animals and microorganisms. Although there have been several studies on the toxoflavin biosynthesis system of *B. glumae*, it is still unclear how *B. gladioli* activates toxoflavin biosynthesis. In this study, we explored the genomic organization of the toxoflavin system of *B. gladioli* and its biological functions using comparative genomic analysis between toxoflavin-producing strains (*B. glumae* BGR1 and *B. gladioli* BSR3) and a strain not producing toxoflavin (*B. gladioli* KACC11889). The latter exhibits normal physiological characteristics similar to other *B. gladioli* strains. *Burkholderia gladioli* KACC11889 possesses all the genes involved in toxoflavin biosynthesis, but lacks the quorum-sensing (QS) system that functions as an on/off switch for toxoflavin biosynthesis. These data suggest that *B. gladioli* has evolved to use the QS signalling cascade of toxoflavin production (TofI/TofR of QS → ToxJ or ToxR → *tox* operons) similar to that in *B. glumae*. However, some strains may have evolved to eliminate toxoflavin production through deletion of the QS genes. In addition, we demonstrate that the toxoflavin biosynthetic system enhances the virulence of *B. gladioli*. These findings provide another line of evidence supporting the differential regulation of the toxoflavin system in *Burkholderia* strains.

Keywords: *Burkholderia gladioli*, *Burkholderia glumae*, genome organization, quorum sensing, toxoflavin, virulence.

INTRODUCTION

Burkholderia is a genus of gram-negative, motile and obligately aerobic rod-shaped proteobacteria (Estrada-de Los Santos *et al.*, 2013). *Burkholderia* species exhibit metabolic versatility and inhabit diverse environments, including soils, plants, animals and humans (Coenye and Vandamme, 2003; Elsas *et al.*, 2002; Estrada-de Los Santos *et al.*, 2001). Although many *Burkholderia*

species are environmentally important and non-virulent, some species can cause diseases in animals and plants. One of these is *B. gladioli*, identified by Lucia McCulloch. In 1921, McCulloch initially reported *B. gladioli* as *Pseudomonas marginata* and *P. gladioli* which caused diseases of gladiolus and iris species (McCulloch, 1921, 1924). *Burkholderia gladioli* can be subdivided into four pathovars: *B. gladioli* pv. *agaricola*, which causes mushroom rot; *B. gladioli* pv. *alliiicola*, which causes onion bulb rot; *B. gladioli* pv. *gladioli*, which causes gladiolus rot; and *B. gladioli* pv. *cocovenenans*, which causes severe food poisoning as a result of the ingestion of infected soybean and coconut-based products (Hildebrand *et al.*, 1973; Jiao *et al.*, 2003; Yabuuchi *et al.*, 1992; Younga *et al.*, 1978). *Burkholderia gladioli* has been reported to cause bacterial panicle blight, as well as sheath and grain browning, in rice in many countries (Nandakumar *et al.*, 2009; Seo *et al.*, 2011; Ura *et al.*, 2006). These symptoms are very similar to those associated with panicle infection by *B. glumae*. Although *B. gladioli* epidemics appear less frequently and this bacterium has relatively weaker virulence than *B. glumae*, these two bacterial species are often isolated from the same infected rice plants (Fory *et al.*, 2014).

In the stationary phase of bacterial growth, the production of a yellow pigment is a representative phenotype of the *B. gladioli* pathovars and *B. glumae*. This unique colour is the result of toxoflavin (1,6-dimethylpyrimido[5,4-e]-1,2,4-triazine-5,7[1H, 6H]-dione (molecular mass, 193; Iiyama *et al.* 1994; Furuya *et al.* 1997; Jeong *et al.* 2003). Several plant pathogens can produce a variety of phytotoxins that are toxic to plant cells and affect disease severity (Durbin, 1991; Geng *et al.*, 2014; Renier *et al.*, 2007). Toxoflavin is a bright yellow phytotoxin that is highly toxic to plants, fungi, animals and microorganisms (Choi *et al.*, 2013; Levenberg and Linton, 1966). Recently, interest in the biosynthesis and genetic organization of toxoflavin is growing because the toxicity of bacterial toxoflavin to plants has led to severe losses in rice crops around the world, including in East Asia, South America and the USA (Ham *et al.*, 2011; Jeong *et al.*, 2003; Kim *et al.*, 2004). Toxoflavin causes chlorotic damage to panicles of plants and inhibits the growth of both leaves and roots of rice (Jeong *et al.*, 2003; Suzuki *et al.*, 1998; Yoneyama *et al.*, 1998). Studies have revealed that toxoflavin performs the following functions: active electron transfer between NADH and oxygen, and the generation of hydrogen peroxide, bypassing the cytochrome system (Latuasan and Berends, 1961; Stern, 1934).

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The genes involved in toxoflavin production can be classified into two groups in *B. glumae*: biosynthesis and transport. These genes are polycistronic. The genes involved in biosynthesis include the five genes *toxABCDE*, and the genes involved in toxoflavin transport include the four genes *toxFGHI* (Kim *et al.*, 2004). These operons are controlled by a TofI (a LuxI-family protein)–TofR (a LuxR-family protein) quorum-sensing (QS) circuit. QS is a cell-to-cell communication mechanism that regulates many physiological systems in *B. glumae*, including flagellar biosynthesis, toxin production and stress responses (Chun *et al.*, 2009; Kim *et al.*, 2007). TofI of the QS system synthesizes the intercellular signalling molecule *N*-octanoyl-homoserine lactone (C8-HSL), which is commonly used by gram-negative bacteria (Kim *et al.*, 2009). C8-HSL binds to its cognate receptor TofR, and the C8-HSL–TofR complex induces the expression of the above-mentioned toxoflavin biosynthetic and transport operons by activating the ToxJ transcriptional regulator. Another regulator, ToxR, also binds toxoflavin as a co-inducer and enhances the expression of the toxoflavin operons (Kim *et al.*, 2004). Genetic and physical evidence indicates that this regulatory system of toxoflavin production in *B. glumae* is organized as a serial cascade (TofI/TofR (QS) → ToxJ or ToxR → *tox* operons; Kim *et al.*, 2004, 2009). Furthermore, Chen *et al.* (2012) have reported recently that the *tofM* mutant strain LSUPB286 produces less toxoflavin than the wild-type strain *B. glumae* 336gr-1, which indicates that TofM, acting as a positive regulator, is very important for toxoflavin production.

However, our understanding of the mechanism of toxoflavin production in *B. gladioli* is at a very rudimentary level. Although it is not difficult to predict the putative functions of genes related to the toxoflavin system through homology searches among DNA sequences, the genomic organization and regulation of the toxoflavin system in *B. gladioli* remain poorly studied. The complete genome sequence of *B. gladioli* BSR3 (Seo *et al.*, 2011) provides information on the genomic organization of the QS and toxoflavin systems. The genomic organization of the genes involved in toxoflavin production is the same in *B. gladioli* BSR3 and *B. glumae* BGR1, except that, in *B. gladioli* BSR3, *toxJ* is located on a different chromosome. The genes encoding the TofI–TofM–TofR QS system in *B. gladioli* BSR3 show the same genome organization as in *B. glumae* BGR1.

In this study, we explored the genes related to toxoflavin biosynthesis in *B. gladioli*. We selected diverse *B. gladioli* strains isolated from various plants, including *B. gladioli* BSR3 (rice), *B. gladioli* KACC11889 (gladiolus), *B. gladioli* KACC13944 (mushroom) and *B. gladioli* KCTC12374 (onion). We used the complete genome sequences of *B. gladioli* BSR3 (Seo *et al.*, 2011) and focused on the genes involved in toxoflavin biosynthesis in *B. gladioli* KACC11889, because this strain does not produce the yellow pigment in the stationary phase. We determined the genomic organization of the toxoflavin system in various *B. gladioli* strains and the role of toxoflavin in the enhanced virulence in

plant hosts. Furthermore, loss of toxoflavin production as a result of a lack of QS genes in *B. gladioli* KACC11889 led to variations in toxoflavin regulation among *Burkholderia* strains.

RESULTS

The toxoflavin biosynthesis of *Burkholderia* species

On the basis of previous studies, we isolated the chemicals secreted by *Burkholderia* strains using thin-layer chromatography (TLC) (Devescovi *et al.*, 2007; Kim *et al.*, 2004). The results of TLC analysis are shown in Fig. 1. Toxoflavin production was visible to the naked eye in most *B. gladioli* strains and in *B. glumae* BGR1. Under UV light at 365 nm, their yellow colouring was even more vivid. *Burkholderia glumae* BGR1 exhibited the strongest yellow colouring, whereas *B. gladioli* BSR3 produced much less toxoflavin. The strains KCTC12374 and KACC13944 produced similar amounts of toxoflavin to that produced by *B. gladioli* BSR3. By contrast, strain KACC11889, which was isolated from gladiolus, did not produce any toxoflavin according to both simple visual inspection and UV light examination. Based on the genome sequence of *B. gladioli* BSR3, this result may indicate that the toxoflavin system was inactive in this strain, possibly at the level of biosynthesis, transport, transcriptional regulation or QS.

Comparative analysis of toxoflavin-related systems

In order to determine whether the toxoflavin system was inactivated in strain KACC11889, we performed a comparative analysis of all gene clusters of the biosynthetic, transport and QS components. First, we determined the nucleotide sequence of the 11 genes in the *tox* operons that are directly responsible for toxoflavin production. These include *toxABCDE* for toxoflavin biosynthesis, *toxFGHI* for toxoflavin transport and *toxJR* for transcriptional regulation (Kim *et al.*, 2004). Using CLUSTALW2 software, we compared all sequence data with genes of *B. glumae* BGR1 to assess their homology. The resulting homology percentages are listed in Table 1. All toxoflavin biosynthetic genes (*toxABCDE*) of *B. gladioli* strains showed very high homology (over 90%) with *B. glumae* BGR1. Similarly, genes of the transporter (*toxFGHI*) showed 89.94%–96.07% homology to the gene in *B. glumae* BGR1, in most cases. Although *toxJ* among transcriptional regulators (*toxJR*) showed a relatively low level of homology, the percentage itself was >80% in all strains. Compared with the genes in strains BSR3, KCTC12374 and KACC13944, the genes in KACC11889 were not notably different (Table 1). In addition, we generated three phylogenetic trees of systems related to toxoflavin production in Phylogeny.fr software (<http://www.phylogeny.fr/>). The data from the phylogenetic trees are shown in Figs S1–S3 (see Supporting Information). All phylogenetic trees indicated that 11 genes of *tox* operons of the

Fig. 1 Toxoflavin production by *Burkholderia*. Five wild-type strains of *Burkholderia* were tested: *B. glumae* BGR1, *B. gladioli* BSR3, *B. gladioli* KACC11889, *B. gladioli* KCTC12347 and *B. gladioli* KACC13944. The supernatant of the bacterial culture was extracted with chloroform and the mixture was centrifuged; the resulting infranant was dried in a centrifugal vaporizer. The solutes were separated in 80% methanol on a thin-layer chromatography (TLC) silica gel plate. The toxoflavin produced was examined visually in daylight and under UV light at 365 nm (T, toxoflavin). The origin (o) and solvent front (f) on the TLC plate are shown.

Table 1 Homology of the *tox* operon between *Burkholderia glumae* BGR1 and *B. gladioli* strains.

Gene	Homology (%)			
	BSR3	KACC11889	KCTC12374	KACC13944
<i>toxA</i>	95.93	95.66	94.71	95.66
<i>toxB</i>	95.78	96.18	95.65	96.05
<i>toxC</i>	95.47	93.26	94.68	93.44
<i>toxD</i>	95.11	94.80	95.51	95.41
<i>toxE</i>	93.77	93.94	93.68	94.11
<i>toxF</i>	95.09	94.44	94.10	95.14
<i>toxF</i>	93.83	93.74	93.93	92.99
<i>toxH</i>	96.07	92.99	95.77	95.48
<i>toxI</i>	91.06	90.53	90.73	89.94
<i>toxJ</i>	83.33	83.27	83.60	83.60
<i>toxR</i>	95.25	95.47	95.47	95.81

strain KACC11889 are very similar to the corresponding genes of the other strains, and there were no significant differences among the homologous sequences.

Subsequently, we characterized the *tofiMR* genes of the QS system, which regulates the operons of toxoflavin biosynthesis (Chen *et al.*, 2012; Kim *et al.*, 2004). The presence of the *tofiMR* genes was confirmed by PCR and electrophoresis. The PCR products are shown in Fig. 2. According to the specific primers used for each gene, the predicted PCR products are 850 bp for the *tofi* (AHL synthase) region, 832 bp for the *tofiM* (quorum-sensing regulatory protein) region and 1267 bp for the *tofiR* (quorum-sensing LuxR family sensor regulator) region. Our results indicate that the strain KACC11889 has no genes similar to *tofiMR*. The outer regions of *tofiM* were not amplified because the agarose gel did not show an ~800-bp PCR band for strain KACC11889 (Fig. 2b). In Fig. 2a,c, PCR bands of sizes that differed from those of the original prod-

ucts were obtained, and sequencing analysis showed that the PCR bands were amplified from unrelated genes. However, the other strains, such as BSR3, KCTC12374, and KACC13944, yielded PCR products of the expected size in all lanes of *tofiMR* electrophoresis. Therefore, we assume that the QS genes required for toxoflavin biosynthesis are inactive in strain KACC11889.

Recovery of toxoflavin biosynthesis in *B. gladioli* KACC11889

To confirm that toxoflavin biosynthesis could be restored by introducing the QS genes, we generated KACC11889 complemented strains in which various QS genes were introduced (*tofi*, *tofiM*, *tofiR* or *tofiMR* from *B. glumae* BGR1 or *B. gladioli* BSR3) following the method described in Experimental procedures. All genes of *B. glumae* BGR1 and *B. gladioli* BSR3 were amplified by PCR and inserted into pBBR1MCS-2 via common restriction sites of the PCR product and the vector (Table S1, see Supporting Information). Genetic validation of the complementation strains involved PCR and DNA sequencing using the PBBR1MCS2_MCS primer set. Eight complementation strains were obtained (Table 2). The complementation strains contained the following introduced genes: 11889ATOFI contained *tofi* from *B. gladioli* BSR3; 11889ATOFM contained *tofiM* from *B. gladioli* BSR3; 11889ATOFR contained *tofiR* from *B. gladioli* BSR3; 11889UTOFI contained *tofi* from *B. glumae* BGR1; 11889UTOFM contained *tofiM* from *B. glumae* BGR1; and 11889UTOFR contained *tofiR* from *B. glumae* BGR1. Furthermore, two additional complementation strains, 11889ATOFIMR and 11889UTOFIMR, were created by introduction of the entire *tofiMR* genetic region from *B. gladioli* BSR3 and *B. glumae* BGR1, respectively.

Fig. 2 Molecular genetic confirmation of the identity of *tof* genes in *Burkholderia gladioli* strains. The *tofIMR* genes were confirmed using polymerase chain reaction (PCR) in four *B. gladioli* strains (BSR3, KACC11889, KCTC12347 and KACC13944). The amplicons were analysed using agarose gel electrophoresis. The positions of the DNA molecular weight markers are indicated to the right of the gels. Black arrows show the position and transcriptional direction of *tofIMR*, and thick bars below the arrows indicate the expected sizes of the PCR products as follows: 850 bp, *tofI*; 832 bp, *tofM*; 1267 bp, *tofR*. (a) The *tofI* gene. (b) The *tofM* gene. (c) The *tofR* gene.

The ability of the complementation strains to produce toxoflavin was evaluated using the above-mentioned TLC assay including wild-type strains (BSR3 and KACC11889). All results were inspected visually in daylight and under UV light at 365 nm. Images of the TLC silica gel plates are shown in Fig. 3a,b. When the TLC silica gel plate with samples of wild-type and complementation strains was developed, the parent strain KACC11889 and the six complementation strains (11889ATOFI, 11889ATOFM, 11889ATOFR, 11889UTOFI, 11889UTOFM and 11889UTOFR) containing the introduced *tofI*, *tofM* and *tofR* genes from *B. gladioli* and *B. glumae*, respectively, did not show any yellow pigment production. However, 11889ATOFIMR and 11889UTOFIMR, which received the entire *tofIMR* from *B. glumae* BGR1 and *B. gladioli* BSR3, respectively, exhibited the production of toxoflavin at a level similar to that of *B. gladioli* BSR3. In addition, these patterns were evaluated on a plate containing a rich medium [Luria–Bertani (LB); Fig. 3c]. 11889EMPTY, which received the empty vector, was used as a negative control. *Burkholderia gladioli* BSR3 and *B. glumae* BGR1 showed abundant production of toxoflavin. Similar to KACC11889, the strain 11889EMPTY did not produce any yellow colouring around the bacterial patch. In contrast, strains 11889ATOFIMR and 11889UTOFIMR fully recuperated from the

loss of the ability to produce toxoflavin. All of these results indicate that inactivation of the toxoflavin system involves the loss of the entire *tofIMR*, and complementation with *tofIMR* can bring about recovery of toxoflavin biosynthesis at a level similar to that in *B. glumae* BGR1 and *B. gladioli* BSR3.

Virulence of the strains with restored toxoflavin production

In order to determine whether recovery of toxoflavin production affected virulence in rice plants, we performed a seed germination assay with wild-type strains (BGR1, BSR3 and KACC11889) and the complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR). Seven days after pre-germination of the seeds, all results were analysed (Fig. 4). In this assay, an LB broth sample served as a control. Strains KACC11889 and 11889EMPTY induced mild disease symptoms, including a slight growth reduction with yellowing (Fig. 4). In contrast, *B. glumae* BGR1 and *B. gladioli* BSR3 caused a severe growth reduction with discoloured stems. Strains 11889ATOFIMR and 11889UTOFIMR were virulent, and the damage caused was similar to that caused by *B. glumae* BGR1 and *B. gladioli* BSR3 (Fig. 4).

Table 2 Bacterial strains and plasmids used in this study.

Name	Description	Source
Bacterial strains		
BGR1	<i>Burkholderia glumae</i> isolate from rice, wild-type	Jeong <i>et al.</i> (2003)
BGS2	BGR1 <i>tofl::Ω</i>	Kim <i>et al.</i> (2004)
BSR3	<i>B. gladioli</i> isolate from rice, wild-type	Seo <i>et al.</i> (2011)
COK94	BSR3 <i>tofl::lacZ</i>	Laboratory collection
KACC11889	<i>B. gladioli</i> isolate from gladiolus, wild-type	KACC
KCTC12374	<i>B. gladioli</i> isolate from onion, wild-type	KCTC
KACC13944	<i>B. gladioli</i> isolate from mushroom, wild-type	KACC
11889EMPTY	KACC11889 transformed with empty vector pBBR1MCS-2	This study
11889ATOFI	KACC11889 transformed with pATOFI	This study
11889ATOFM	KACC11889 transformed with pATOFM	This study
11889ATOFR	KACC11889 transformed with pATOFR	This study
11889ATOFIMR	KACC11889 transformed with pATOFIMR	This study
11889UTOFI	KACC11889 transformed with pUTOFI	This study
11889UTOFM	KACC11889 transformed with pUTOFM	This study
11889UTOFR	KACC11889 transformed with pUTOFR	This study
11889UTOFIMR	KACC 11889 transformed with pUTOFIMR	This study
DH5α λpir	<i>Escherichia coli</i> used for cloning and propagation of plasmids	Laboratory collection
S17-1 λpir	<i>E. coli</i> used for analysis of conjugal transfer functions	Kalogeraki and Winans (1997)
Plasmids		
pBBR1MCS-2	Broad-host-range vector containing mobilization gene and kanamycin resistance gene	Kovach <i>et al.</i> (1995)
pATOFI	pBBR1MCS-2 containing <i>bglA</i> _2g11050 (<i>tofl</i>) gene of BSR3	This study
pATOFM	pBBR1MCS-2 containing <i>bglA</i> _2g11060 (<i>toflM</i>) gene of BSR3	This study
pATOFR	pBBR1MCS-2 containing <i>bglA</i> _2g11070 (<i>toflR</i>) gene of BSR3	This study
pATOFIMR	pBBR1MCS-2 containing <i>bglA</i> _2g11050-70 (<i>toflMR</i>) genes of BSR3	This study
pUTOFI	pBBR1MCS-2 containing <i>bglu</i> _2g14490 (<i>tofl</i>) gene of BGR1	This study
pUTOFM	pBBR1MCS-2 containing <i>bglu</i> _2g14480 (<i>toflM</i>) gene of BGR1	This study
pUTOFR	pBBR1MCS-2 containing <i>bglu</i> _2g14470 (<i>toflR</i>) gene of BGR1	This study
pUTOFIMR	pBBR1MCS-2 containing <i>bglu</i> _2g14470-90 (<i>toflMR</i>) genes of BGR1	This study

KACC, Korean Agricultural Culture Collection (<http://www.genebank.go.kr>); KCTC, Korea Collection Type Cultures (<http://kctc.kribb.re.kr>).

Furthermore, the enhanced virulence of the complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR) was in agreement with the results of the onion tissue maceration assay (Fig. 5). After inoculation, the area of macerated tissue was monitored for 72 h. The strains KACC11889 and 11889EMPTY yielded 0–1 cm of macerated tissue, whereas the complemented toxoflavin-producing strains 11889ATOFIMR and 11889UTOFIMR yielded macerated areas >2 cm and severe water-soaking symptoms. Thus, the complemented toxoflavin-producing strains were able to cause severe damage to host plants as a result of the introduction of the QS genes from *B. glumae* BGR1 or *B. gladioli* BSR3.

Swarming motility of *Burkholderia*

Swarming motility is a typical phenotypic trait of QS-related downstream systems. *Burkholderia glumae* BGR1 is able to move about using flagella to reach the site of infection in host plants (Kim *et al.*, 2007). We tested the swarming motility of wild-type strains (BGR1, BSR3 and KACC11889), QS-deficient mutants (BGS2, *B. glumae* *tofl* mutant; COK94, *B. gladioli* *tofl* mutant) and the complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR). All results are shown in Fig. 6. The wild-type strain BGR1 showed swarming motility on 0.5% agar plates,

whereas the mutant BGS2 (*tofl::Ω* mutant strain) did not show any movement (Kim *et al.*, 2007). Likewise, *B. gladioli* BSR3 exhibited swarming motility, whereas the mutant COK94 (*tofl::lacZ* mutant strain) did not (Kim *et al.*, 2014). The strain KACC11889 exhibited normal swarming motility on 0.5% agar plates. The complemented toxoflavin-producing strains exhibited swarming motility similar to that of the wild-type strain KACC11889.

DISCUSSION

Plant-pathogenic bacteria produce diverse phytotoxins, including albicidin, coronatine, fervenulin, reumycin and toxoflavin (Geng *et al.*, 2014; Levenberg and Linton, 1966; Renier *et al.*, 2007). Albicidin produced by *Xanthomonas albilineans* causes the chlorotic symptoms of leaf scald disease in sugarcane by blocking chloroplast development (Renier *et al.*, 2007). Numerous strains of *Pseudomonas syringae* produce the phytotoxin coronatine, which is a multifunctional virulence factor that induces leaf chlorosis, hypertrophic growth on potato and inhibition of root growth in rice (Geng *et al.*, 2014; Mittal and Davis, 1995). Among the above-mentioned toxins, toxoflavin is potent in plants, fungi, animals and microorganisms, and acts as an azapteridine antibiotic (Fenwick *et al.*, 2011; Levenberg and Linton, 1966; Sato *et al.*, 1989). As a prosthetic group, toxoflavin acts as an effective electron carrier

Fig. 3 Restoration of toxoflavin production by genetic complementation of strains of *Burkholderia gladioli* KACC11889. (a) Thin-layer chromatography (TLC) analysis in complementation strains receiving the *tof* genes of *B. gladioli* BSR3. Four complementation strains were tested: 11889ATOFI, 11889ATOFM, 11889ATOFR and 11889ATOFIMR. *Burkholderia gladioli* BGR3 and KACC11889 are indicated to the left of the plates as a control. The toxoflavin produced was evaluated visually in daylight and under UV light at 365 nm (T, toxoflavin). The origin (o) and solvent front (f) on the TLC plate are shown. (b) TLC analysis of the complementation strains that received the *tof* genes of *B. glumae* BGR1. Four complementation strains were tested: 11889UTOFI, 11889UTOFM, 11889UTOFR and 11889UTOFIMR. (c) The pigmentation phenotype of the complementation strains 11889ATOFIMR and 11889UTOFIMR. *Burkholderia gladioli* BSR3 and *B. glumae* BGR1 were used as positive controls. The strain 11889EMPTY, which was created by introduction of the empty vector pBBR1MCS-2, served as a negative control. Freshly grown bacterial culture was spread on a Luria–Bertani (LB) agar plate and photographed after 48 h of incubation at 30 °C post-inoculation.

and helps to produce superoxide and hydrogen peroxide in the presence of oxygen and light (Latusan and Berends, 1961; Stern, 1934). In plants, this toxic property of toxoflavin is essential for the inhibition of elongation of sprouts and roots and for the induction of grain rot in rice (Suzuki *et al.*, 1998; Yoneyama *et al.*, 1998). To date, there have been several studies of the toxoflavin biosynthesis system, especially in *B. glumae*. The toxoflavin biosynthesis system contains two polycistronic operons: a biosynthetic operon (*toxABCDE*) and a transport operon (*toxFGHI*) (Kim *et al.*, 2004). It has also been reported that the toxoflavin biosynthesis system is controlled by the QS system (Kim *et al.*, 2009), depending on the homoserine lactone (HSL) synthesized by TofI and the cognate receptor TofR, by activating ToxJ and ToxR as

transcriptional regulators of toxoflavin biosynthesis. It is still unclear how *B. gladioli* activates the toxoflavin biosynthesis system. Therefore, we focused on whether toxoflavin-related operons are present in and show homology among the genomes of *B. gladioli* strains, and whether the *B. gladioli* strains utilize the general cascade (TofI/TofR of QS → ToxJ or ToxR → *tox* operons) of toxoflavin biosynthesis of *B. glumae*.

In this study, we tested *B. gladioli* BSR3 isolated from rice; the genome sequence of this strain is well known (Seo *et al.*, 2011). We also evaluated three representative strains of the main pathovars: *B. gladioli* KACC11889, a strain isolated from gladiolus; *B. gladioli* KACC13944, a strain isolated from mushrooms; and *B. gladioli* KCTC12374, a strain isolated from onions. Using

(a)

(b)

Fig. 4 Virulence of the complemented toxoflavin-producing strains in rice according to changes in seed germination. (a) Damage to rice seeds. Pre-germinated seeds of rice were inoculated with the bacterial culture [optical density at 600 nm (OD_{600}) of 0.5], as described in Experimental procedures. The LB liquid medium was used as a negative control. The photographs were taken 7 days after inoculation. (b) The length of stems. Average length of the inoculated seeds was measured/calculated 7 days after inoculation. Each bar shows the standard error from five replicates.

Fig. 5 Virulence of the complemented toxoflavin-producing strains in onion, judged by tissue maceration. Onion tissue samples were inoculated with the wild-type strain (KACC11889), negative control (11889EMPTY) and the complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR). All samples were incubated at 30 °C for 72 h. The photographs were taken 3 days after inoculation.

TLC analysis, we showed that all strains, except KACC11889, produced the yellow pigment toxoflavin, which was visible to the naked eye and under UV light (Fig. 1). However, KACC11889 showed normal growth during all experiments. These data provided a very useful starting point to conduct comparative analysis between a functional and defective toxoflavin system for the identification of genes that activate toxoflavin biosynthesis in *B. gladioli*. First, we predicted that the defective toxoflavin system of strain KACC11889 was the result of the loss of either the biosynthetic or the transport operon, because it has been reported that mutations in these operons result in the loss of the ability to produce toxoflavin (Suzuki *et al.*, 2004). Nevertheless, the biosynthetic operon *toxABCDE*, the transport operon *toxFGHI* and the transcriptional regulators *toxJR* of strain KACC11889 showed

very high sequence homologies to those of *B. gladioli* BSR3 (Table 1 and Figs S1–S3). However, the genetic defect in strain KACC11889 is located in the QS system (*toflMR*) of chromosome 2 of *B. gladioli* BSR3 (Fig. 2). The QS system is a cell-to-cell communication mechanism and regulates a diverse range of bacterial behavioural patterns, including the formation of biofilms, flagellar biosynthesis, stress responses and the production of virulence factors (Chun *et al.*, 2009; Miller and Bassler, 2001). Kim *et al.* (2004) reported that a QS-deficient mutant (BGS2) produces neither the HSL signalling molecule nor toxoflavin. According to these data, we believe that *B. gladioli* has evolved to utilize the general cascade (TofI/TofR of QS → ToxJ or ToxR → *tox* operons) of *B. glumae*, as well as to selectively accommodate the original QS system, serving as an on/off switch of toxoflavin biosynthesis. Furthermore, a complemented strain of *B. gladioli* KACC11889, in which *tofl/R* from *B. gladioli* BSR3 was introduced, did not produce any toxoflavin that was detectable in the liquid medium or on a TLC plate (Fig. S5, see Supporting Information). This result supports the function of TofM as a positive regulator of toxoflavin biosynthesis in *B. glumae* 336gr-1 (Chen *et al.*, 2012).

It should be noted that *B. gladioli* BSR3 contains another QS system (*bgla_1p1740-60*), which is located on plasmid 1 (Choudhary *et al.*, 2013). Previously, we have reported the total gene expression profile of *B. gladioli* BSR3 in the exponential phase and stationary phase using RNA sequencing (Kim *et al.*, 2014). We confirmed that the QS system (*bgla_1p1740-60*) on plasmid 1 yields stronger responses to population density than the other QS genes (*bgla_2g11050-70*) on chromosome 2 (Fig. S4, see Supporting Information). Furthermore, the *tox* genes of *B. gladioli* are divided between two chromosomes (the *toxJ* gene is in chromosome 2, whereas the others are in chromosome 1), in contrast with the neighbouring genes in chromosome 2 of *B. glumae* (Lim *et al.*, 2009; Seo *et al.*, 2011). These observations indicate that *B. gladioli* may utilize a specialized or dichotomous regulatory mechanism through two QS systems. Moreover, the present experimental results add weight to our ideas relating to the regulation of the two QS genes. The introduction of the *tof* genes from *B. glumae* BGR1 or from *B. gladioli* BSR3 restores toxoflavin biosynthesis in strain KACC11889 (Fig. 3). This restoration, which is mediated by the *tof* genes of *B. glumae*, indicates that *B. gladioli* also uses QS-mediated toxoflavin biosynthesis in a similar manner to that in *B. glumae*. In addition, there is systemic flexibility, which may change according to environmental factors, via the loss of the on/off switch of the QS system rather than inactivation of the biosynthesis system itself. In other words, *B. gladioli* has evolved the ability to utilize a regulatory system different from the strong regulation produced by the original QS mechanism. The swarming motility of strain KACC11889 is a good illustrative example. Kim *et al.* (2007) reported that the mutated *tofl* strain BGS2 shows significantly reduced swimming motility and no swarming motility. In contrast (Fig. 6), we observed active

Fig. 6 Swarming motility of *Burkholderia*. A swarming motility assay was performed to confirm bacterial motility in three wild-type strains (*B. glumae* BGR1, *B. gladioli* BSR3 and KACC11889), two quorum-sensing (QS)-deficient strains (*B. glumae* BGS2 and *B. gladioli* COK94) and two complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR, derived from KACC11889). The cells were seeded onto 0.5% Luria–Bertani (LB) agar plates and photographed after 24 h of incubation at 30 °C.

swarming motility in strain KACC11889, just as in *B. glumae* BGR1 and *B. gladioli* BSR3, on 0.5% agar plates. Thus, this phenomenon could be explained by the presence of a different regulatory system in strain KACC11889. In addition, the QS-deficient *B. glumae* mutant strains (deletion mutants of *tofl* or *tofr*) produced toxoflavin on LB agar medium (Chen *et al.*, 2012). This indicates that *B. glumae* and *B. gladioli* have diverse regulatory systems for toxoflavin production through QS.

What are the other possible functions of the toxoflavin biosynthesis system in plant-pathogenic *Burkholderia* strains? As an azapteridine antibiotic, toxoflavin is toxic to other microorganisms and also causes stress to the producer itself. For example, it has been reported that the growth of *Candida albicans* is inhibited by farnesol (Shirtliff *et al.*, 2009). Farnesol is a well-known virulence factor and QS signalling molecule in *C. albicans* (Cells *et al.*, 2008; Hornby *et al.*, 2001). Hence, strain KACC11889 might have evolved to use virulence factors other than toxoflavin. Indeed, Suzuki *et al.* (2004) found that a toxoflavin-deficient mutant strain of *B. glumae* is still able to cause damage to rice seedlings and rice panicles. These data indicate that toxoflavin is a very important virulence factor, but that *Burkholderia* exhibits a complex pathogenesis mechanism because of multiple virulence factors. In contrast, toxoflavin biosynthesis confers enhanced virulence in rice to

B. gladioli KACC11889. We also found that the complemented toxoflavin-producing strains (11889ATOFIMR and 11889UTOFIMR, derived from KACC11889) inflict a greater severity of damage than the parental strain on rice and onion (Figs 4 and 5).

In conclusion, we have characterized the genomic organization of the toxoflavin system and the different modes of regulation for toxoflavin biosynthesis in *B. gladioli*. In addition, we have demonstrated that the capacity for toxoflavin biosynthesis confers enhanced virulence to this phytopathogen. Our findings provide another line of evidence supporting the differential regulation of the toxoflavin system in *Burkholderia* strains. Further studies should lead to a better understanding of the global regulatory system, which involves the double QS systems of *B. gladioli*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, oligonucleotide primers and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 2. The PCR primers used in this study are shown in Table S1. A single colony grown on an LB agar plate was incubated in liquid medium in a shaker-incubator at 200 rpm. The culture temperature for all strains was 30 °C.

TLC analysis

Each strain was cultured in a 2-mL microcentrifuge tube containing 1.5 mL of LB broth at 30 °C for 1 day. Then, 1 mL of the liquid culture was pelleted by centrifugation at 12 000 *g* for 1 min; 500 µL of the supernatant was transferred into a new tube containing 500 µL of chloroform. After one round of vortexing, the mixture was again pelleted by centrifugation at 12 000 *g* for 10 min. Next, 500 µL of the infranant was transferred to a new tube and dried in a centrifugal vaporizer (Tokyo Rikakikai, Japan). The solutes were then dissolved in 10 µL of 80% methanol, and 1 µL of the solution was spotted onto a TLC silica gel plate (Merck Millipore, Darmstadt, Germany). This step was repeated 10 times. The TLC silica gel plate was placed in a TLC chamber (the solvent was chloroform–methanol, 95: 5) to start chromatography. 3UV™ Multi-Wavelength lamps (UVP, Upland, CA, USA) were used to examine the band pattern on the TLC silica gel plate.

DNA sequencing and data analysis

Primer sets for PCR and sequencing are listed in Table S1. The reaction mixture for PCR was as follows: 5 µL, 10 × Pfu buffer; 1 µL, deoxynucleoside triphosphates (dNTPs) (10 mM stock); 2 µL, Primer 1 (10 pmol/µL); 2 µL, Primer 2 (10 pmol/µL); 0.5 µL, Pfu DNA polymerase (SolGent, Seoul, South Korea); 10 µL, 5X Band Doctor™; *x* µL (~10 ng), template; *y* µL (up to the total volume of 100 µL), distilled water. PCR was performed under the following conditions: one cycle of 10 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 53 °C and 2 min at 72 °C. After an electrophoresis step, the amplified fragment was extracted from the gel using the AccuPrep® PCR Purification Kit (Bioneer, Daejeon, South Korea). DNA sequencing was performed by MacroGen Inc., using the 3730xl DNA analyser (Applied Biosystems®, Waltham, Massachusetts, USA). The DNA sequence data were analysed using CLUSTALW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) and Phylogeny.fr (<http://www.phylogeny.fr/>).

Creation of transformant strains

To create each transformant clone, general and standard techniques were used for DNA manipulations, cloning, restriction digestion and agarose gel electrophoresis (Sambrook *et al.*, 1989). Genes of *B. gladioli* BSR3 and *B. glumae* BGR1 were amplified by means of Pfu DNA polymerase. To insert a gene into the pBBR1MCS-2 vector, restriction sites were added to both PCR primers. Table S1 shows the restriction sites of all primers. The amplified fragment and pBBR1MCS-2 were digested using appropriate restriction enzymes to obtain common restriction sites and were ligated using DNA ligase. *Escherichia coli* DH5α *λpir* competent cells were transformed with the resulting vector, and the transformed cells were cultured in a kanamycin-containing (50 µg/mL) medium. To confirm correct ligation, we extracted plasmid DNA from the cells and performed restriction digestion and electrophoresis. In the conjugation procedure, the properly assembled plasmid DNA was again used for the transformation of *E. coli* S17-1 *λpir*, and the resulting bacterial cells were mated with the strain KACC11889. Transformant cells that were grown on a plate with two antibiotics (tetracycline, 20 µg/mL; kanamycin, 50 µg/mL) were collected and the genetic composition was confirmed by PCR with two primers designed to bind outside the multiple cloning site of pBBR1MCS-2 (Table S1).

Seed germination assay

Each strain was incubated in 2 mL of LB liquid medium at 30 °C for 12–14 h with shaking, and then subcultured at 30 °C until an optical density at 600 nm (OD₆₀₀) of 0.5 was reached in 5 mL of LB liquid medium. Seeds of rice (*Oryza sativa* L. cv. Dongjin) were pre-germinated at 30 °C in a bacterial suspension. Two days later, the germinating seeds were transferred to a sterile plant culture dish (SPL Life Sciences, Pocheon, South Korea) containing 20 mL of distilled water, and were grown for 7 days under the following conditions: 30 °C, high relative humidity (close to 100%), 16 h of light provided by 400-V lamps, during the day; 28 °C, high relative humidity (close to 100%), 8 h of darkness, at night. The damage to and length of rice seeds were assessed 7 days after inoculation. The experiments were repeated five times.

Onion tissue maceration assay

All papery skins and external layers of onions were removed. An onion was quartered with a sterilized knife. Onion scales were placed in sterilized plant culture dishes (SPL Life Sciences) containing 5 mL of distilled water, and each onion scale was wounded on the inner surface by a sterile 1–10-µL pipette tip. Then, 10 µL of bacterial culture adjusted to OD₆₀₀ = 0.5 was placed onto the wound of the onion scale. All samples were incubated at 30 °C for 72 h. Tissue maceration was assessed 3 days after inoculation.

Bacterial swarming motility assay

This assay was performed using LB broth in agar (0.5%, w/v). Each strain was incubated at 30 °C for 12–14 h in 2 mL of LB liquid medium. Then, 1 mL of cultured cells was pelleted by centrifugation at 900 *g* for 2 min. The harvested cells were washed with 1 mL of fresh LB liquid medium, and the centrifugation step was repeated. The washed pellet was resuspended in 100 µL of distilled water, and 1 µL of the suspension was spotted onto the assay plate. The assay plate was incubated at 30 °C for 24 h.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Fig. S1 The phylogenetic tree of toxoflavin biosynthesis genes in *Burkholderia*. Five wild-type strains of *Burkholderia* were analysed: *B. glumae* BGR1, *B. gladioli* BSR3, *B. gladioli* KACC11889, *B. gladioli* KCTC12347 and *B. gladioli* KACC13944. The phylo-

genetic tree of *toxABCDE* was constructed using Phylogeny.fr software. The bootstrap consensus was inferred from 1000 replicates showing values of >30.

Fig. S2 The phylogenetic tree of toxoflavin transport genes in *Burkholderia*. Five wild-type strains of *Burkholderia* were analysed: *B. glumae* BGR1, *B. gladioli* BSR3, *B. gladioli* KACC11889, *B. gladioli* KCTC12347 and *B. gladioli* KACC13944. The phylogenetic tree of *toxFGHI* was constructed using Phylogeny.fr software. The bootstrap consensus was inferred from 1000 replicates showing values of >30.

Fig. S3 The phylogenetic tree of toxoflavin transcriptional regulator genes in *Burkholderia*. Five wild-type strains of *Burkholderia* were analysed: *B. glumae* BGR1, *B. gladioli* BSR3, *B. gladioli* KACC11889, *B. gladioli* KCTC12347 and *B. gladioli* KACC13944. The phylogenetic tree of *toxIR* was constructed using Phylogeny.fr software. The bootstrap consensus was inferred from 1000 replicates showing values of >30.

Fig. S4 Expression of quorum sensing (QS) genes in *Burkholderia gladioli* BSR3. Expression levels of two QS systems (*bglA*_{2g11050-70} and *bglA*_{1p1740-60}) were measured using RNA sequencing in *B. gladioli* BSR3. The results were calculated as RPKM (reads per kilobase of exon per million mapped sequence reads). Blue and red bars indicate the exponential phase and stationary phase, respectively.

Fig. S5 Toxoflavin production and reverse transcription-polymerase chain reaction (RT-PCR) in a *tofl/R*-complemented strain (11889ATOFIR) of *Burkholderia gladioli* KACC11889. (a) Thin-layer chromatography (TLC) analysis of toxoflavin production in 11889ATOFIR, in which the *tofl/R* genes of *B. gladioli* BSR3 were introduced. *Burkholderia gladioli* BGR3 and KACC11889 are shown on the right side of the plates as controls. Toxoflavin production was evaluated visually in both daylight and under UV light at 365 nm (T, toxoflavin). The origin (o) and solvent front (f) on the TLC plate are indicated. (b) RT-PCR analysis of the *toflMR* genes. The expression of the *toflMR* genes was confirmed using RT-PCR of total RNA from four *B. gladioli* strains (BSR3, KACC11889, 11889ATOFIR and 11889ATOFIR). The 16s ribosomal RNA was used as a positive control. The amplified products were 225 bp for *tofl* (lane 1: primers 5'-GTACCTGCTGCAC GAGGTG-3' and 5'-CGCTCGATACTGCAGAAGGT-3'), 201 bp for *toflM* (lane 2: primers 5'-TTTCGCACACCTGACCGTT-3' and 5'-GTGAGGGCCTGGTTGACC-3'), 208 bp for *toflR* (lane 3: primers 5'-CTGGATGGCCCATTACCAGG-3' and 5'-GATGGTCAGCAGTC CGAACA-3') and 282 bp for the control (lane 4: primers 5'-CCAGCAGCCGCGGTAATACG-3' and 5'-TACCAGGGTATCTAA TCC-3').

Table S1 PCR primers used in this study.