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Characterization of Three Types of Quorum-Sensing Mutants in *Burkholderia glumae* Strains Isolated in Japan

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Abstract

Quorum sensing (QS) has been believed to be essential for the production of virulence factor such as toxoflavin, oxalate and the motility in *Burkholderia glumae*. Two types of QS mutant (*tofI* mutant) were recently reported in geographically different isolates of *B. glumae*. The *tofI* mutant derived from *B. glumae* BGR1 isolated in Korea had lost toxoflavin production but not the one derived from *B. glumae* 336gr-1 isolated in the United States. In the present study, we generated *tofI* mutants from 14 different *B. glumae* strains isolated in Japan and investigated the QS-regulated phenotypes. All *tofI* mutants failed to produce *N*-acyl-$_L$-homoserine lactones. However, *tofI* mutants from 11 out of 14 strains (Group I) retained toxoflavin productivity both on a solid and in a liquid medium like *B. glumae* 336gr-1 *tofI* mutant, whereas those from 2 strains (Group II) lost toxoflavin productivity both on a solid and in a liquid medium similar to *B. glumae* BGR1 *tofI* mutant. The other strain (Group III) showed novel phenotype because toxoflavin production was observed only on a solid medium. Furthermore, Group II *tofI* mutants lost oxalate productivity and exhibited a severe reduction in motility while Group I *tofI* mutants slightly produced oxalate and showed higher motility than Group II *tofI* mutants. These data suggest that the difference between Group I and II is not limited to toxoflavin production but also observed other QS-regulated functions. On the other hand, R1-types of morphological mutants frequently emerge from S-type colonies during the subculture for *B. glumae* (Tsushima et al., 1991). This study revealed that Group I wild-type strains displayed the S-type morphology and those of Group II showed the R1-type morphology. The results suggest that colony type is involved in the QS-regulated functions of Group I and II *tofI* mutants.

Keywords: *Burkholderia glumae*, quorum-sensing mutant, colony morphological mutant

1. Introduction

Burkholderia glumae is responsible for bacterial seedling rot and grain rot disease of rice and this pathogen causes serious damage to rice production in several countries (Goto & Ohata., 1956; Cottyn et al., 1996a; Cottyn et al., 1996b; Jeong et al., 2003; Zeigler & Alvares., 1989). The pathogenicity of *B. glumae* is associated with its multiplication on the grain after translocation from the leaf sheath (Tsushima., 1996; Tsushima et al., 1987). The *tofI*/*tofR* quorum-sensing (QS) system of *B. glumae* plays a central role in its pathogenicity (Kim et al., 2004). QS is a gene regulation system depending on cell density in bacteria (Fuqua et al., 1996). In several gram-negative bacteria including *B. glumae*, *N*-acyl-$_L$-homoserine lactone (AHL) is used as a QS signal molecule in this system. AHL is constitutively synthesized by a member of the LuxI protein family and the concentration increases with local cell-density. When AHL concentration reaches a threshold, they form complex with the AHL receptor protein belonging to the LuxR protein family and bind certain sequence called *lux*-box like sequence, resulting in coordinated various gene expressions. In *B. glumae*, *tofI* encodes a AHL synthase responsible for the production of *N*-octanoyl-$_L$-homoserine lactone (C8-HSL) and *N*-hexanoyl-$_L$-homoserine lactone (C6-HSL) while *tofR* encodes a AHL receptor. Among them, C8-HSL has been identified as the main QS signal molecule in *B. glumae*. *B. glumae* produces phytotoxins including fervenulin, reumycin and toxoflavin (Kim et al., 2004). In the *B. glumae* BGR1 strain isolated in Korea,

disruption of *tofI* resulted in the loss of toxoflavin production because the C8-HSL-TofR complex activates *toxJ*, which is responsible for the expression of the *tox* operon required for toxoflavin biosynthesis and secretion (Kim et al., 2004). Furthermore, QS regulates the expression of the gene encoding an IclR-type transcriptional regulator, *qsmR* (Kim et al., 2007), which regulates oxalate production (Goo et al., 2012), catalase activity (Chun et al., 2009), motility (Kim et al., 2007) and the type II secretion system (Goo et al., 2011). Because QS is essential for rice spikelet pathogenicity in *B. glumae* BGR1 (Kim et al., 2007), it is expected to become one of the main targets for antivirulence therapy and to replace the current antimicrobials for *B. glumae*. However, the *tofI* mutant of *B. glumae* 336gr-1, isolated in the United States, retains a high level of toxoflavin productivity and pathogenicity on spikelets, despite the loss of AHL productivity (Chen et al., 2012). Although it is unknown what cause the discrepant results between BGR1 and 336gr-1, it will become an important research for *B. glumae* to understand phenotypic and genetic diversity among strains. Since BGR1 and 336gr-1 are geographically distinct isolates, we focussed on the role of QS in *B. glumae* strains isolated in Japan because bacterial seedling rot and grain rot disease of rice by *B. glumae* was first reported in Japan (Uematsu et al., 1976; Goto & Ohata., 1956). In this study, we constructed and characterized the QS mutants of the *B. glumae* strains isolated in Japan.

2. Materials and Methods

2.1 Bacterial Strains, Plasmids and Chemicals

We used 14 *B. glumae* strains isolated from various sites on rice or bean sprouts at several locations in Japan from 1979 to 2005 (Table 1). The other bacterial strains and plasmids used in this study are listed in Table 2. Luria-Bertani (LB) medium was used as the routine bacterial growth medium (Sambrook et al., 1989) and potato semisynthetic agar (PSA) medium (Yoshida et al., 2001) was used to study colony morphology Both C6-HSL and C8-HSL were synthesized as previously described (Ikeda et al., 2001). Antibiotics were added with final concentrations of 100 μg/ml ampicillin, 50 μg/ml kanamycin and 10 μg/ml collistin.

2.2 Construction of the tofI Mutants

We constructed a marker-less *tofI* mutant by site-directed mutagenesis using inverse PCR (Dorrell et al., 1996). An approximately 2300-bp sequence containing the *tofI* region was amplified by PCR using KOD FX polymerase (Toyobo Life Science, Japan). The forward and reverse primers *tofI*-1000-F (5′-TGACGTCGCGATCCTCTGTCTGC-3′) and *tofI*-1000-R (5′-GAGCTGCTGCTCGTTTTCCGCC-3′), respectively, were designed based on the *B. glumae* BGR1 genome (Lim et al., 2009). Chromosomal DNA extracted from MAFF 302748 served as a template under the following reaction conditions: 94 °C for 2 min, followed by 27 cycles at 98 °C for 10 s and 68 °C for 2.5 min. The PCR fragment was inserted into the pGEM-T Easy Vector using TArget Clone -Plus- (Toyobo Life Science, Japan) and Ligation high Ver2 (Toyobo Life Science, Japan) to generate pMtofI1000. Furthermore, we designed two primers in opposite orientations at the mutation site: *tofI*-N, 5′-CATTCGCATCGCGTCCCAATACGT-3′ and *tofI*-C, 5′-TCACGTTCTGCAGCATCGAGCG-3′. Inverse PCR was performed with the primer set, KOD FX polymerase and pMtofI1000 as template under the following reaction conditions: 94 °C for 2 min, followed by 10 cycles at 98 °C for 10 s and 68 °C for 5 min. After amplification, the remaining plasmid DNA template was removed by digestion with DpnI and self-ligated to generate the pMtofI800 vector. The kanamycin resistance cassette was cut out of the pKPR11 vector using PstI and introduced into the PstI site of the pGP704Sac38 suicide vector, which contains sucrose-sensitive gene *sacB*, to generate pGPSacBKm. The pMtofI800 vector was digested with EcoRI and the fragment containing the disrupted *tofI* was inserted into the MfeI site of pGPSacBKm to generate pGPSacBtofI800. The disruption of *tofI* was performed by bacterial conjugation and homologous recombination. Conjugation was conducted between each strain of *B. glumae* and *Escherichia coli* S17-1 λpir carrying pGPSacBtofI800. After incubation in LB agar containing 10 mM $MgSO_4$ at 30 °C for 4 h, the cells were streaked on LB agar containing 50 μg/ml kanamycin and 10 μg/ml collistin (all *B. glumae* strain tolerant to collistin) and incubated at 37 °C for 48 h. This medium is sensitive for *E. coli* S17-1 λpir but resistant for *B. glumae*. Single colonies were picked up with a toothpick, transferred to 4 ml of LB broth and incubated at 37 °C for 16 h with shaking. After incubation, the culture was streaked on LB agar plates containing 10% sucrose and incubated at 37 °C for 24 h. The disruption of *tofI* was confirmed by AHL productivity and by the band size of the PCR product amplified with the tofI (H)-F primer 5′-GTTCGTCAACGACGACTACG-3′ and tofI (H)-R primer 5′-CGGAATTACCACGAGGACAC-3′ (Chen et al., 2012).

2.3 Detection of AHL Productivity

We investigated the different types of AHL produced by the wild-type strains using thin-layer chromatography (Shaw et al., 1997) using TLC plate (RP-18 F254S, Merck) and *C. violaceum* CV026 as a biosensor, which

clearly distinguishes C6-HSL and C8-HSL (McClean et al., 1997). The AHL production by *tofI* mutants was investigated by cross-streak using *C. violaceum* CV026 and VIR07 (Morohoshi et al., 2008). *C. violaceum* VIR07 shows stronger responses to C8-HSL but not to C6-HSL than CV026. These biosensors response to exogenous AHLs by producing the purple pigment violacein.

2.4 Characterization of the QS-Regulated Functions

We investigated toxoflavin and oxalate productivity as well as the motility of the 14 wild-type strains and mutants. Toxoflavin appears as a yellow pigment in LB medium (Chen et al., 2012; Kim et al., 2004). Therefore, each strain was streaked on LB agar plates with or without 100 nM C8-HSL and incubated at 37 °C for 24 h. Toxoflavin productivity was assessed by the intensity of the yellow pigment in solid culture. Furthermore, we investigated toxoflavin productivity in liquid culture. Fourty microlitter of each cell suspension (OD_{610} = 0.2) was inoculated to 4 ml of LB broth and incubated at 37 °C for 24 h with shaking. After incubation, toxoflavin was extracted from culture supernatant with equal volume of chloroform and dried in draft chamber for a night. The extraction was resuspended in 100 µl of methanol and toxoflavin productivity was assessed by the intensity of the yellow pigment. For the oxalate assay, 5 µl of each cell suspension was added to LB agar containing 0.1% $CaCl_2$ (Li et al., 1999) with or without 100 nM C8-HSL, and incubated at 37 °C for 24 h. Oxalate productivity was positive when calcium oxalate crystals were observed in the medium using light microscopy (Olympus SZ61, Olympus Corporation, Tokyo, Japan). Motility was investigated as previously described, with some modifications (Kato et al., 2013). Each cell suspension was deposited at the centre of a 90 φ × 15 mm petri dish containing 0.3% LB agar and incubated at 37 °C for 48 h. Motility was investigated three times and assessed by measuring colony diameter.

2.5 Colony Morphology

We investigated colony morphology of the wild-type strains on PSA medium. The colony type was divided into S-type (smooth, white, shiny, dome) or R1-type (rough, translucent, flat), as previously described (Tsushima et al., 1991).

Table 1. *Burkholderia glumae* strains isolated in Japan

Burkholderia glumae	Isolation date	Source	Site on the source	Location
MAFF 301441	1982	Oryza sativa	Unknown	Hiroshima, Japan
MAFF 302417	1984	Vigna radiate	Sprout	Tokyo, Japan
MAFF 302437	1984	Oryza sativa	Grain	Ooita, Japan
MAFF 302552	1991	Oryza sativa	Leaf sheath	Kumamoto, Japan
MAFF 302748	1982	Oryza sativa	Seedling	Ibaraki, Japan
MAFF 302874	1979	Oryza sativa	Grain	Ibaraki, Japan
MAFF 302930	1990	Oryza sativa	Seedling	Iwate, Japan
MAFF 302934	1990	Oryza sativa	Seedling	Miyagi, Japan
MAFF 311026	1991	Oryza sativa	Seedling	Hokkaido, Japan
MAFF 311193	1997	Oryza sativa	Seedling	Toyama, Japan
MAFF 311196	1996	Oryza sativa	Seedling	Nagano, Japan
MAFF 311199	1996	Oryza sativa	Seedling	Yamaguchi, Japan
MAFF 311266	1982	Oryza sativa	Grain	Fukuoka, Japan
MAFF 311509	2005	Oryza sativa	Grain	Okinawa, Japan

Table 2. Bacterial strains and plasmids

Strains	Description	Source or reference
Escherichia coli		
S17-1 λ/pir	*thi pro hsdR hsdM*$^{+}$ *recA* RP4 2Tc::Mu-Km::Tn7	Simon et al., 1983
Burkholderia glumae		
dI441	A Δ*tofI* derivative of MAFF 301441	This study
dI417	A Δ*tofI* derivative of MAFF 302417	This study
dI437	A Δ*tofI* derivative of MAFF 302437	This study
dI552	A Δ*tofI* derivative of MAFF 302552	This study
dI748	A Δ*tofI* derivative of MAFF 302748	This study
dI874	A Δ*tofI* derivative of MAFF 302874	This study
dI930	A Δ*tofI* derivative of MAFF 302930	This study
dI934	A Δ*tofI* derivative of MAFF 302934	This study
dI026	A Δ*tofI* derivative of MAFF 311026	This study
dI193	A Δ*tofI* derivative of MAFF 311193	This study
dI196	A Δ*tofI* derivative of MAFF 311196	This study
dI199	A Δ*tofI* derivative of MAFF 311199	This study
dI266	A Δ*tofI* derivative of MAFF 311266	This study
dI509	A Δ*tofI* derivative of MAFF 311509	This study
Chromobacterium violaceum		
CV026	ATCC 31532 derivative, *cviI*::Tn5sylE, Km^{r}, Sm^{r}	McClean et al., 1997
VIR07	ATCC 12472 derivative, *cviI*:: Km^{r}, Amp^{r}	Morohoshi et al., 2008
Plasmids		
pGEM-T easy	Cloning vector, Amp^{r}	Promega
pMtofI1000	pGEM-T containing *tofI,* Amp^{r}	This study
pMtofI800	pGEM-T containing *ΔtofI*, Amp^{r}	This study
pKRP11	Cloning vector, Km^{r}, Amp^{r}	Reece & Phillips., 1995
pGP704Sac38	pBR322 derivative with R6K *ori*, *mob* RP4, polylinker from M13 tg131 containing *sacB*, Amp^{r}	Morohoshi et al., 2004
pGPSacBKm	pGP704Sac38 inserted Km^{r} gene	This study
pGPSacBtofI800	pGPSacBKm containing *ΔtofI*, Km^{r}	This study

3. Results

3.1 tofI Disruption Prevents AHL Production

We characterized the different types of AHLs produced by each *B. glumae* strain and confirmed that all the wild-type strains produced C8-HSL and C6-HSL (Table 3). Furthermore, we constructed a marker-less *tofI* mutant for each strain and confirmed that all *tofI* mutants failed to produce AHLs (Table 3). The size of the PCR fragment amplified with tofI (H) primer sets was as expected (data not shown).

Table 3. Productivity of AHL, toxoflavin, oxalate and colony morphology for *Burkholderia glumae* wild-type strains isolated in Japan and their *tofI* mutants

	AHL productivity	Toxoflavin productivity		Oxalate productivity	Colony morphology	Group
		Solid culture	Liquid culture			
MAFF 301441	C6-HSL, C8-HSL	+	+	++	S[a]	I
dI441	-	+	+	+	ND[b]	
MAFF 302417	C6-HSL, C8-HSL	+	+	++	R1[c]	II
dI417	-	-	-	-	ND	
MAFF 302437	C6-HSL, C8-HSL	+	+	++	S	I
dI437	-	+	+	+	ND	
MAFF 302552	C6-HSL, C8-HSL	+	+	++	S	I
dI552	-	+	+	+	ND	
MAFF 302748	C6-HSL, C8-HSL	+	+	++	S	I
dI748	-	+	+	+	ND	
MAFF 302874	C6-HSL, C8-HSL	+	+	++	R1	II
dI874	-	-	-	-	ND	
MAFF 302930	C6-HSL, C8-HSL	+	+	++	S	I
dI930	-	+	+	+	ND	
MAFF 302934	C6-HSL, C8-HSL	+	+	++	S	III
dI934	-	+	-	+	ND	
MAFF 311026	C6-HSL, C8-HSL	+	+	++	S	I
dI026	-	+	+	+	ND	
MAFF 311193	C6-HSL, C8-HSL	+	+	++	S	I
dI193	-	+	+	+	ND	
MAFF 311196	C6-HSL, C8-HSL	+	+	++	S	I
dI196	-	+	+	+	ND	
MAFF 311199	C6-HSL, C8-HSL	+	+	++	S	I
dI199	-	+	+	+	ND	
MAFF 311266	C6-HSL, C8-HSL	+	+	++	S	I
dI266	-	+	+	+	ND	
MAFF 311509	C6-HSL, C8-HSL	+	+	++	S	I
dI509	-	+	+	+	ND	

[a] S-type colony morphology [b] Not determined [c] R1-type colony morphology.

3. 2 Strain-Specific QS Dependency for Toxoflavin and Oxalate Production

All the wild-type strains possessed toxoflavin production both on a solid and in a liquid medium (Table 3). As the result of *tofI* disruption, among the marker-less *tofI* mutants, 11 out of 14 strains (Group I) retained toxoflavin productivity both on a solid medium and in a liquid medium similar to *B. glumae* 336gr-1 (Figure 1A, B). Two strains, dI417 and dI874 (Group II) lost the ability to produce toxoflavin both on a solid medium and in a liquid medium, like *B. glumae* BGR1 (Figure 1A, B). Furthermore, dI934 (Group III) was able to produce toxoflavin on solid medium but not in liquid medium (Figure 1A, B).

3. 3 Evidence for QS Dependent and Independent Motility

The Group I and III *tofI* mutants showed equal level of or slightly lower motility with that of their wild-type strains (Figure 3A). The loss of toxoflavin and oxalate productivity in the Group II *tofI* mutants was associated with a severe reduction in motility compared with their wild-type strains and Group I and III *tofI* mutants (Figure 3A, B). The Group II wild-type strains showed greater motility than that of the Group I and III wild-type strains (Figure 3B), suggesting that other factors affect motility. Furthermore, the addition of C8-HSL dramatically restored the motility in the dI874 mutant of Group II (Figure 3C).

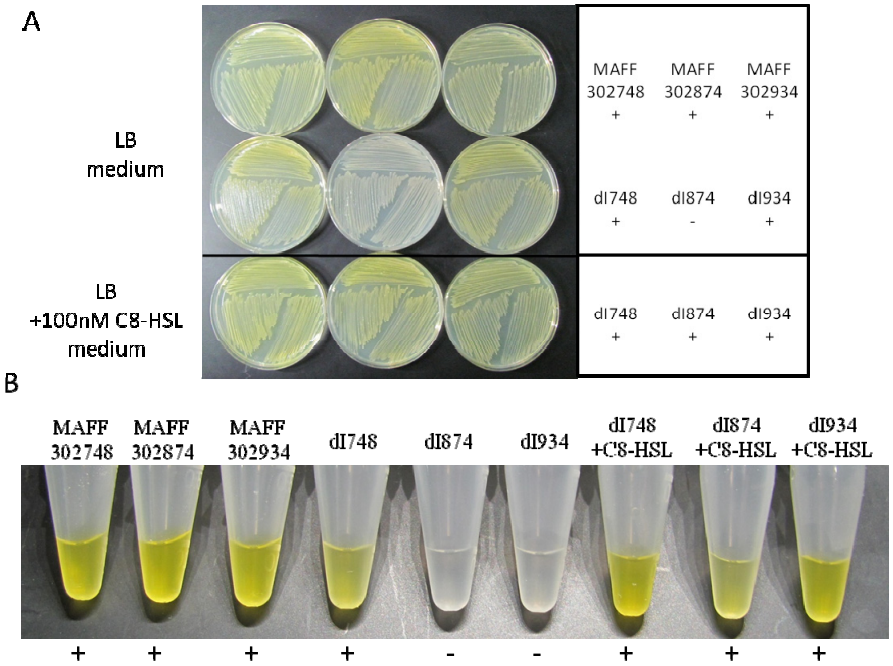

Figure 1. Toxoflavin productivity of the wild-type strains MAFF 302748,MAFF 302874, MAFF 302934 and their respective *tofI* mutants dI748,dI874 and dI934 on LB agar (A) or in LB broth (B). Each strain was streaked on LB agar or cell suspension (OD_{610}=0.2) was inoculated to LB broth and incubated at 37 °C for 24 h with or without 100 nM C8-HSL. After incubation, toxoflavin productivity was assessed by the productivity of yellow pigment in agar or concentrated chloroform extraction from liquid culture

All the wild-type strains exhibited oxalate productivity (Table 3). As the result of *tofI* disruption, oxalate calcium crystals were observed in Group I and III but not detected in Group II (Table 3, Figure 2). The amount of oxalate for Group I and III *tofI* mutants appeared to be lower than that for the wild-type strain. This is because oxalate calcium crystals were observed inside and outside the wild-type colonies but only inside the *tofI* mutant colonies (Figure 2, Table 3). The addition of C8-HSL restored toxoflavin and oxalate productivity in the dI874 mutant of Group II (Figures 1A, B and 2), essentially bypassing the loss of *tofI*-dependent AHL synthase expression.

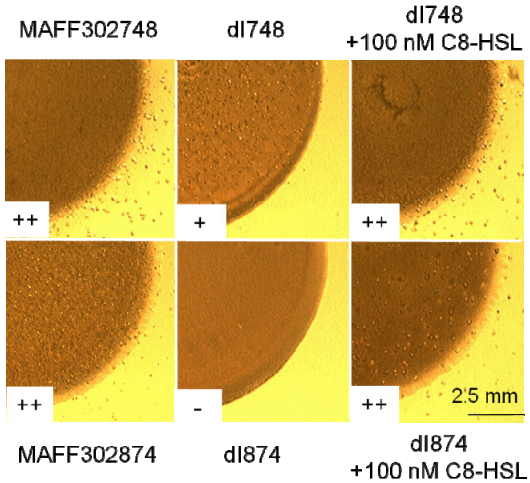

Figure 2. Oxalate productivity of the wild-type strains MAFF 302748 and MAFF 302874 and their respective *tofI* mutant dI748 and dI874. Each strain was grown on LB agar containing 0.1% $CaCl_2$ with or without 100 nM C8-HSL at 37 °C for 24 h. Oxalate productivity was detected by the presence of calcium oxalate crystals in inside and/or outside of the colony using light microscopy

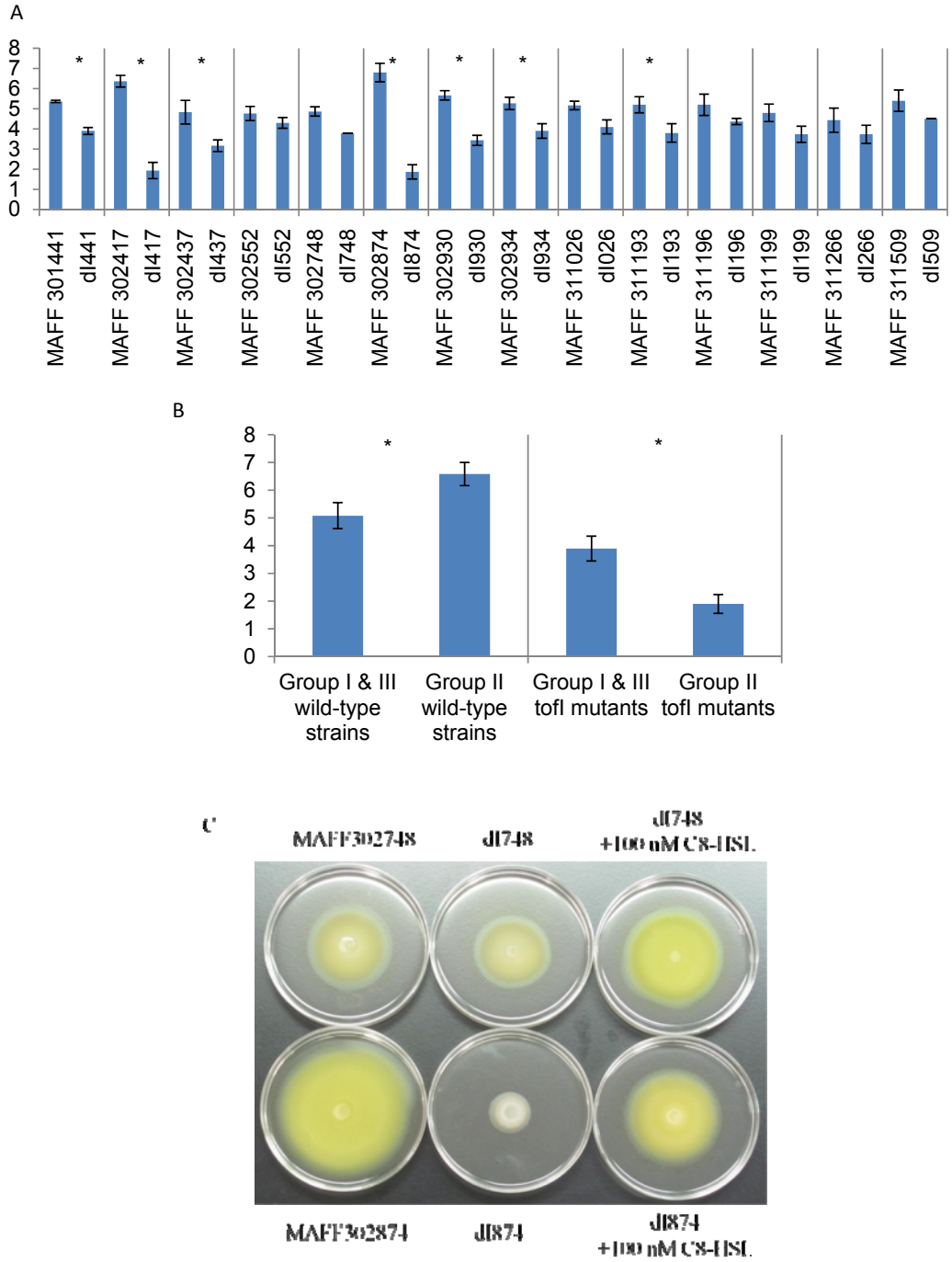

Figure 3. Motility of *B. glumae* wild-type strains isolated in Japan and respective *tofI* mutants on 0. 3% LB agar medium with or without 100 nM C8-HSL at 37 °C for 48 h. (A) Comparison of motility between the wild-type strain and *tofI* mutant. (B) Comparison of average of colony diameter between Group I, III and II wild-type strains or *tofI* mutants. Bars on columns indicate that standard deviations. Differences in the motility were determined by Tukey–Kramer multiple comparisons method. * indicates significant differences between each strain or groups (P, 0.01). (C) Motility of the wild-type strains MAFF 302748 and MAFF 302874 and respective *tofI* mutant dI748 and dI874 with or without 100 nM C8-HSL

3.4 Different Colony Morphology Among Group I and II Wild-Type Strains

All Group I and III wild-type strains showed smooth, white, shiny and dome-type colony morphology (S-type colony) (Figure 4). On the other hand, we could not detect the S-type colony in Group II wild-type strains.

Instead, they presented rough, translucent and flat colony morphology (Figure 4), which is typical of the R1-type colony that emerged during subculture of the S-type strain of *B. glumae* (Tsusima et al., 1991).

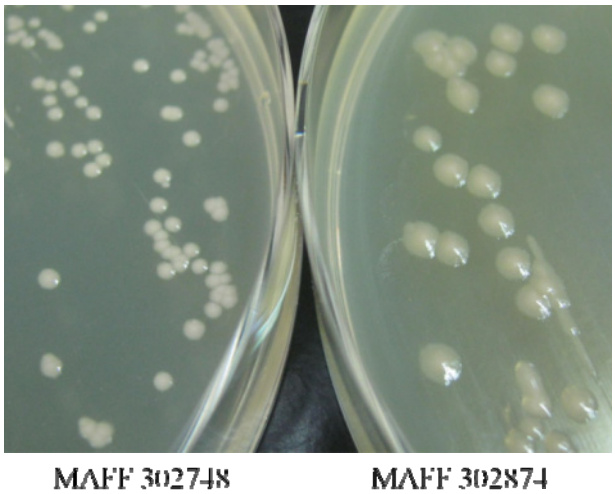

Figure 4. Colony morphology of the wild-type strains MAFF 302748 and MAFF 302874. Each strain was streaked on potato semisynthetic agar (PSA) medium and incubated at 30°C for four days

4. Discussion

Two *B. glumae* QS mutants with distinct pathogenicity were reported in Korea (*B. glumae* BGR1; Kim et al., 2004) and the United States (*B. glumae* 336gr-1; Chen et al., 2012). Although both presented mutations in the *tofI* gene, which is responsible for toxoflavin production, *B. glumae* 336gr-1 retained toxoflavin-related pathogenicity. The present study was designed to investigate the causes of this discrepancy to improve our understanding of the regulation mechanisms of virulence-related functions and strain diversity for *B. glumae*.

We constructed and characterized 14 QS *tofI* mutant *B. glumae* strains isolated from various regions in Japan. As expected, all QS mutants failed to produce AHLs (Table 3). However, 12 mutant strains (Group I and III) maintained toxoflavin productivity on a solid medium like the American *B. glumae* 336gr-1 strain, whereas two strains (Group II) lost the productivity similar to Korean *B. glumae* BGR1 (Table 3). These data suggested the existence of *tofI*-independent mechanisms for the regulation of toxoflavin, as previously suggested for 336gr-1 (Chen et al., 2012). Furthermore, not only Group II *tofI* mutant but also Group III dI934 did not produce toxoflavin in liquid culture (Table 3, Figure 1B). Although we reisolated dI934 from liquid culture and inoculated them to solid medium, they retained toxoflavin productivity on solid medium (data not shown), suggests that the loss of toxoflavin production by dI934 is specific to liquid culture. The amount of toxoflavin produced by *qsmR* mutant increases until 12 h after incubation but then decrease after 16 h in liquid medium but not on solid medium (Kim et al., 2007). Thus, it will be necessary to investigate the relationship between incubation times and toxoflavin productivity. In addition, we demonstrated that other QS-regulated functions, such as oxalate production and motility, of the *tofI* mutants were still preserved on the equal or slightly lower level with the wild-type strains in Group I and III but severe reduction of these functions was observed in Group II (Table 3, Figures 3A, B). Toxoflavin production is directly regulated by QS (Kim et al., 2004), whereas oxalate production and motility are indirectly regulated by QS via QsmR (Goo et al., 2012; Kim et al., 2007). Therefore, unknown additional regulators may be involved in not only toxoflavin production but also other QS-regulated functions in Group I and III.

The addition of C8-HSL was able to recover the functions of Group II and III *tofI* mutant to almost equal level with the wild-type strain (Figures 1, 2, 3C), suggesting that secondary mutations in other important sites for these functions during allelic-exchange procedure as previously observed in *Pseudomonas aeruginosa* QS mutants (Beaston et al., 2002) is not occurred.

Phylogenetic analysis based on *gyrB* and *rpoD* sequences revealed a limited diversity of the Japanese strains of *B. glumae* compared with other *Burkholderia* plant pathogens and that the two members of Group II (MAFF 302417 and MAFF 302874) were in the same cluster as Group I strains, MAFF 302552 (Maeda et al., 2006).

This indicates that the differences between Group I and II are not associated with phylogenetic associations based on housekeeping genes.

For several years, QS has been considered to be essential for the expression of virulence-related functions and for the plant and human pathogenicity of *Burkholderia* species (Kim et al., 2004; Ulrich et al., 2004). A recent study showed that the *B. mallei* GB8 and ATCC 23344 QS mutant exhibited a pathogenicity comparable with that the parent strain in an aerosolized mouse infection model (Majerczyk et al., 2013) contrary to the previous report in other *B. mallei* QS mutant (Ulrich et al., 2004). As one possibility, the authors suggested that laboratory passages and the accumulation of spontaneous mutations select these different types of QS mutants because the genome of *B. mallei* is plastic (Nierman et al., 2004) and genome variability between passages is considered to be a feature of *B. mallei* (Romero et al., 2006). Similarly, natural mutants of *B. glumae* frequently emerge during subculture and rice infection (Tsushima et al., 1991; Kato et al., 2013; Nandakumar et al., 2009). It is reported that there are three types of colony morphological mutants (CMMs) from S-type colony strains of MAFF 302748 during subculture: R1-1, R1-2 and R2 (Kato et al., 2013). In this study, all members of Group I and III wild-type strains showed an S-type colony morphology, whereas both Group II wild-type strains showed an R1-type colony morphology (Figure 4). There are possibilities that the Group II strains experienced genomic mutations during laboratory passage. However, the previous isolates of CMM showed lower motility than the wild-type strain (Kato et al., 2013), whereas the Group II strains were extremely motile (Figure 3C). Thus, the previous CMM isolates and the Group II wild-type strains may represent different types although they share the R1-type colony morphology.

The commercial type strain ATCC 33617 of *B. glumae* lost its toxoflavin productivity and pathogenicity on rice spikelets because of frameshift mutations in *tofR* (Devescovi et al., 2007). *tofR* complementation restored toxoflavin production and pathogenicity, which suggests that type strain ATCC 33617 may belong in Group II. However, additional mutations may exist because mutation sites are not limited to one place in laboratory strains (Beaumont et al., 2009).

Mutable functions not associated with the phylogenetic relationship are common features of *B. glumae*, which makes it difficult to elucidate the mechanisms supporting virulence. It will be important to understand phenotypic and genetic diversity among inter-strains in future studies for *B. glumae*. Although the relationship between colony morphological change and the generation of Group I and II remains poorly understood, it may be necessary to consider the generation of intra-strain variation to shape strain diversity for *B. glumae*.

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