농학박사학위논문

세균벼알마름병원균 *Burkholderia glumae*의 정치배양시 발생하는 적응 돌연변이와 펠리클 형성

Adaptive mutation and pellicle formation in static culture of *Burkholderia glumae* causing bacterial grain rot

2021년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

곽 지 영

Adaptive mutation and pellicle formation in static culture of *Burkholderia glumae* causing bacterial grain rot

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by

Gi-Young Kwak

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN PLANT MICROBIOLOGY

Adaptive mutation and pellicle formation in static culture of *Burkholderia glumae* causing bacterial grain rot

UNDER THE DIRECTION OF DR. INGYU HWANG

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
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Adaptive mutation and pellicle formation in static culture of *Burkholderia glumae* causing bacterial grain rot

Gi-Young Kwak

Abstract

Bacteria under unfavorable environment make necessary adjustments to survive. The morphological and genetic diversification in bacteria involve changes including lifestyle switch from motile to sessile cells in pellicle initiation and development. The pellicle as a thin buoyant multicellular layer at the air-liquid interface in Gram-negative bacteria have received little attention compared to the solid surface colonizing biofilm produced from Gram-negative bacteria. Though, there remain questions to be answered in functional role and the trigger of bacterial cellulose biosynthesis as pellicles, here the aerobic rice pathogen *Burkholderia glumae* BGR1 biosynthesizing cellulase-sensitive pellicles in bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)- and flagellum-dependent, yet in quorum sensing independent manner with colony morphological variation and reduced virulence is shown. The elevated level of c-di-GMP was detected in pellicle forming colony variants. Since c-di-GMP is involved in adhesin production and motility associated biofilm formation inhibition, the PAS/GGDEF motif possessing diguanylate cyclase gene for c-di-GMP diosynthesis, the *pelI* from *B. glumae* and the adapted GGDEF motif carrying response

regulator, *pleD*, from *Agrobacterium tumefaciens* was taken to investigate a role in facilitated pellicle formation in *B. glumae* under unfavorable *in vitro* condition. A cluster of genes involved in bacterial cellulose biosynthesis (BCS) was found to harbor *bcsD*, *bcsR*, *bcsQ*, *bcsA*, *bcsB*, *bcsZ*, and *bcsC* in *B. glumae*. An inverse relationship between the mutations in BCS genes and virulence of BCS mutants in rice plants was observed that the mutants had significantly reduced virulence. The *bcsB* upregulated PAS/PAC domain carrying *bspP* (BGLU_RS28885) mutant strain in *B. glumae* had facilitated pellicle formation. The quicker pellicle formation was observed from *bspP* spontaneous deletion or point mutation strains of *B. glumae.* The spontaneous mutations in *bspP* was considered as genetic sacrifice to better adapt to unfavorable environment and to survive. By possessing relatively flexible genome structures as survival tactics in bacteria, the aerobic BGR1 genetically alters genotype resulting phenotypic changes that fits the environment better. The colonies with various morphology and quicker pellicle formation as more fitted individuals were able to survive a week in static culture growth condition. The unfavorable bacterial niche such as omitted aeration from *in vitro* culture condition for an aerobe, triggered colony variants with diverse genetic mutations in *bspP* to facilitate pellicle formation for aerobic cells to gain excess to available oxygen. The cells in static culture were able to survive as they avoid cell death due to alkalization via the *obcA* upregulation for keeping extracellular pH close to neutral through oxalate production. Hence, the extended survivorship in *bspP* mutant was supported by *bcsB* gene upregulation, and oxalate-mediated detoxification of the alkaline static environment. Though *bspP* as adaptive genetic provided successful extension in viability in unfavorable environment, had adverse effects on *B. glumae* colonization and virulence in the host.

KEY WORDS: *Burkholderia glumae*, Pellicle, Bacterial lifestyle switch, Virulence, Colony variants, Bacterial adaptation

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INTRODUCTION

Bacterial adaptation to constantly changing environment to survive incorporates genetic flexibility in acquiring beneficial genetic traits and phenotypes (Francino, 2012). Bacterial survivorship through necessary alterations in their genetic organization, behavior, and life style changes involve bacterial biofilm formation as a result of evolution in unfavorable growth condition. Bacterial biofilms are multicellular complexes composed of self-producing extracellular polysaccharides, proteins, and nucleic acids (Donlan, 2002; Flemming et al., 2007).

The biofilms in general, develop on diverse solid surfaces and the biofilms that occupy the air-liquid interface as a floating thin bacterial cellulosic multicellular layer is called pellicles (Armitano et al., 2014). In unfavorable environment or certain ecological niches, bacterial cells form complex biofilm structures for their best chance of survival (Williams and Cannon, 1989; Kovács and Dragoš, 2019).

The motile cells move in flagella dependent manner in search of better microniche for their survival. In addition, many cellular regulations for survival such as bacterial colonization, pathogenesis, and motility are known to be QS dependent (Kim et al., 2004, 2007). Though the QS wild-type strain of *Burkholderia glumae*, BGR1, flagella dependent motility is regulated by QS

that it also contributes to symptom development in rice plants, pellicle is formed in QS-independent manner (Kwak et al., 2020).

The pellicle is a multicellular layer that colonizes the air-liquid interface of static culture when the bacterial culture is incubated without agitation (Armitano et al., 2014). *B. glumae* as aerobic bacterium requires oxygenated culture condition, which is provided by 250 rpm in laboratory liquid culture condition. The aerobic bacterial cells search for available oxygen in oxygen depleted condition and naturally explore their surroundings and reach the air-liquid interface by flagellum mediated motility (Römling et al., 2005, 2013).

The bacterial flagellum mediated motility is then switched to sessile cells once the bacterial cells successfully reach the air-liquid interface where the atmospheric oxygen is most abundantly available through bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)-mediated signal transduction systems (Römling and Amikam, 2006). This bacterial lifestyle switches from motile to sessile cells are triggered by unfavorable culture condition in the laboratory that in unfavorable niche, bacterial cells form biofilm structures for their survival (Williams and Canon, 1989; Kovács and Dragoš, 2019). C-di-GMP is biosynthesized by diguanylate cyclases (DGCs) that possesses a GGDEF motif and hydrolysed into 5′-phosphoguanylyl-(3′-5′)-guanosine by phosphodiesterases (PDEs) that carries an EAL domain and degraded by

proteins containing HD-GYP motif (Valentini and Filloux, 2016).

The main component of the pellicles in Gram-negative bacteria is the cellulose and the proteobacteria inhabiting diverse ecological niches comprises cellulose producers (Speirs et al., 2003; Ude et al., 2006; Hölscher et al., 2015; O'Toole et al., 2000). This bacterial cellulosic pellicle is produced by five main proteins encoded by bacterial cellulose biosynthase (BCS) genes, *bcsA*, *B*, *C*, *D*, and *Z* (Saxena et al., 1994; Le Quéré and Ghigo, 2009). In synchrony with BCS genes, c-di-GMP plays a role in regulation of biofilm formation (Hengge, 2009; Römling et al., 2013).

In unfavorable colonizing conditions such as omitted aeration from the aerobic bacteria culture condition, pellicle is triggered and facilitated, in non-kinetic sense, that bacterial genomic flexibility plays a role in physiological adjustment for their survival (Francino, 2012). The genomic flexibility that allows bacteria to adapt to unfavorable environment provides advantages in bacterial evolution and helps us to better understand the bacterial responses to environmental cues in nature for their survival (Francino, 2012; Giri et al., 2019; Rainey et al., 1998, 2017; Martin et al., 2016). The spatially constructed environment that generate heterogenous experimental bacteria culture condition as static culture is conceived as oxygen-deficient environment for aerobic bacteria (Spiers et al., 2003; Morris et al., 2014, Lind et al., 2015). The microniche with constant consumption of available oxygen and nutrients

within the spatially restricted space as static culture, is where the aerobic bacteria experience selection pressure under the unaerated static culture environments and forces them to evolve to maximize their survival (D' Souza et al., 2014; Koskiniemi et al., 2012; Frenoy and Bonhoeffer, 2018).

I used the rice bacterial pathogen *B. glumae* to investigate how the bacterium genetically and physiologically adapts to unfavorable environment such as oxygen depleting culture conditions. *B. glumae* uses amino acids as carbon sources and releases ammonia as a result of deamination that causes an alkaline environmental pH that leads to cell death (Goo et al., 2017). Hence detoxification becomes essential for the *B. glumae* survival when grown in nutrient rich media (Goo et al., 2012). Hence, I suggest the survivorship of pellicle forming mutants under static culture condition evolved ability to bypass the cell death due to alkalization.

Based on my previous findings in pellicle formation extending survivorships in *B. glumae* in oxygen-limiting static culture (Kwak et al., 2020), I investigated further into how *B. glumae* cells are genetically and physically altered in pellicle-forming static culture environment. Hence, I additionally address the pellicle formation accompanied with colony variants as one of physiological alteration in bacteria in making efforts to best fit the disadvantageous environment, which results from the genetic alteration of *bspP* (BGLU_RS28885). The *bspP* putatively encodes a protein possessing

Per-Arnt-Sim (PAS) and bacterial stage II sporulation E (SpoIIE) domains, which aids the survival in static culture condition.

I also suggest the genetic mutations may be a sacrifice for the benefits of a whole bacterial population under selection pressure and facing adaptation to survive, because the stressed aerobic bacterial population in *in vitro* static culture condition lead to the evolutionary process as the cells produce quicker pellicles for their survival. The acquired survivorship via the facilitated pellicle formation and colony variants lead to a rather surprising outcome in the host rice plants. The unexpected consequence was that the advantageous pellicle formation as *in vitro* evolutionary adaptation lead to extended survivorship but abolished virulence and adversely affected colonization in *in vivo* condition. This clearly indicate that the genetic and phenotypic adaptive alteration for the *in vitro* survival might not always be advantageous in host plants colonization.

Considering the aerobic plant pathogenic bacteria, *B. glumae* infecting the host plant tissues and being able to proliferate after successful invasion, the physiological conditions of apoplast in an interaction with the pellicle forming BGR1 mutant in rice plant is worth noting in understanding the unsuccessful colonization and becoming an avirulence strain.

Here, a suggestion is made with evidence of advantages of pellicles that are formed in unfavorable *in vitro* environment that extended survivorship was given to *B. glumae* in an exchange of genetic sacrifice. I also suggest, the

pellicle formation as an evolutionary consequence may significantly decrease or abolish virulence in *B. glumae*. These findings intrigue us to explore further into the interaction between the stress preadapted pathogen and host plant.

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CHAPTER 1

Quorum Sensing-Independent Cellulase-Sensitive Pellicle Formation is Critical for Colonization of *Burkholderia glumae* in Rice Plants

ABSTRACT

Bacteria form biofilms as a means to adapt to environmental changes for survival. Pellicle is a floating biofilm formed at the air–liquid interface in static culture conditions; however, its functional roles have received relatively little attention compared to solid surface-associated biofilms in Gram-negative bacteria. Here the rice pathogen *Burkholderia glumae* BGR1 forming cellulase-sensitive pellicles in a bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)- and flagellum-dependent, but quorum sensing (QS)-independent, manner is shown. Pellicle formation was more favorable at 28°C than at the optimum growth temperature (37°C), and was facilitated by constitutive expression of *pelI*, a diguanylate cyclase gene from *B*. *glumae*, or *pleD*, the GGDEF response regulator from *Agrobacterium tumefaciens*. Constitutive expression of *pelI* or *pleD* raised the levels of c-di-GMP, facilitated pellicle formation, and suppressed swarming motility in *B*. *glumae*. QS-defective mutants of *B*. *glumae* formed pellicles, while flagellum-defective mutants did not. Pellicles of *B*. *glumae* were sensitive to cellulase but not to proteinase K or DNase I. A gene cluster containing seven genes involved in bacterial cellulose biosynthesis, *bcsD*, *bcsR*, *bcsQ*, *bcsA*, *bcsB*, *bcsZ*, and *bcsC*, homologous to known genes involved in cellulose biosynthesis in other bacteria, was identified in *B*. *glumae*. Mutations in each gene abolished pellicle

formation. These results revealed a positive correlation between cellulase-sensitive pellicles and putative cellulose biosynthetic genes. Pellicle-defective mutants did not colonize as successfully as the wild-type strain BGR1 in rice plants, which resulted in a significant reduction in virulence. My findings show that cellulase-sensitive pellicles produced in a QS-independent manner play important roles in the interactions between rice plants and *B*. *glumae*.

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INTRODUCTION

Bacterial biofilms are complex multicellular complexes embedded with self-producing extracellular materials such as polysaccharides, proteins, and nucleic acids (Donlan, 2002; Flemming et al., 2007). Bacterial biofilms are generally developed on diverse solid surfaces, and biofilm formed at the air–liquid interface is called floating biofilm or pellicle (Armitano et al., 2014). Under unfavorable growth conditions or in certain ecological niches, bacterial cells form complex biofilm structures for their survival (Williams and Cannon, 1989; Kovács and Dragoš, 2019). Aerotactic bacterial cells are coagulated at the air–liquid interface by flagellum-mediated motility, then switch their lifestyles to become sessile cells through bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)-mediated signal transduction systems (Römling et al., 2005, 2013; Römling and Amikam, 2006). In addition, bacterial quorum sensing (QS) often plays critical roles in biofilm formation (De Kievit, 2009; Guttenplan and Kearns, 2013; Hentzer et al., 2004). Formation of pellicles provides fitness and survival advantages (Boles and Singh, 2008); however, the roles of cellulosic pellicles in the natural environment are not well understood, especially in interactions between plant pathogenic bacteria and their hosts.

Common features of pellicles in Gram-negative bacteria include that oxygen is critical to triggering pellicle formation, flagellar motility is important,

and cellulose is a main component of the pellicle matrix (Hölscher et al., 2015; Ude et al., 2006; Spiers et al., 2003; Barken et al., 2008). One variety of Proteobacteria inhabiting diverse ecological niches comprises bacterial cellulose producers (O'Tool et al., 2000; Sutherland, 2001). Bacterial cellulose facilitates intimate interactions between bacterial cellulose producers and various components present in the environment (Yamamoto et al., 2011). Particularly for plant- or animal-associated bacteria, cellulosic biofilms are important for close interactions with their hosts (Pérez-Mendoza et al., 2014; Augimeri et al., 2015; Yang et al., 2019). Formation of bacterial cellulosic pellicle is accomplished by five main proteins encoded by bacterial cellulose biosynthetic (bcs) genes, *bcs A*, *B*, *C*, *D*, and *Z* (Saxena et al., 1994; Le Quéré and Ghigo, 2009). In addition to cellulose biosynthetic genes, c-di-GMP plays a key role in the regulation of bacterial biofilm formation (Hengge, 2009; Houry et al., 2010; Römling et al., 2013). C-di-GMP is biosynthesized by diguanylate cyclases (DGCs) that often possess a GGDEF motif, and then hydrolyzed into 5′-phosphoguanylyl-(3′-5′)-guanosine by phosphodiesterases (PDEs) carrying an EAL and degraded by proteins containing a HD-GYP motif (Valentini and Filloux, 2016; Ryan et al., 2009). These proteins interplay to produce and degrade c-di-GMP to maintain its proper concentration under given environmental conditions. In general, c-di-GMP stimulates biosynthesis of adhesins and inhibits various forms of motility associated with the switch from a motile planktonic lifestyle to a sedentary biofilm-associated lifestyle,

whereas overproduction of EAL domain proteins induces motility (Jenal and Malone, 2006; Simm et al., 2004; Römling et al., 2005, 2013).

The rice pathogenic bacterium *Burkholderia glumae*, which causes panicle blight was used, to study functional roles of biofilm upon interaction with rice plants. Panicle blight is a rice disease that causes serious economic losses when weather conditions are favorable for the pathogen (Kim et al., 2004, 2007). The *B. glumae* forming cellulase-sensitive pellicles in static culture was found, then investigation of the factors involved in pellicle formation and determination of their functional roles in interactions between rice plants and *B. glumae* were made. One of the diguanylate cyclase genes present in the genome of *B. glumae* was identified to exhibit the most influence on pellicle formation. Heterologous expression of the *pleD* gene (Atu1297), whose protein is a response regulator possessing the GGDEF domain in *Agrobacterium tumefaciens*, facilitated pellicle formation in *B. glumae*. Pellicle formation was dependent upon temperature, flagella, and c-di-GMP, but independent of QS. The pellicles playing critical roles in colonization of *B. glumae*, thereby affecting virulence in rice plants are shown here.

MATERIALS AND METHODS

I. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. glumae* and *Escherichia coli* were grown at 37°C and 250 rpm in Luria-Bertani (LB) broth containing 0.1% tryptone, 0.5% yeast extract, and 0.5% NaCl (w/v) (USB, Cleveland, OH, USA). When necessary, appropriate antibiotics were added as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; trimethoprim, 75 μg/ml; rifampicin, 100 μg/ml; spectinomycin, 100 μg/ml. LB agar medium contained 1.5% (w/v) agar (Becton Dickinson, Sparks, MD, USA).

II. Nucleic acid manipulations

The methods for DNA cloning, restriction mapping, and gel electrophoresis was done in a standard procedure (Sambrook et al., 2001). DNA was treated with the appropriate restriction enzymes as instructed by the supplier (New England Biolabs, Ipswich, MA, USA), and extraction of the DNA fragments from the gels was carried out as described by the manufacturer (Qiagen, Valencia, CA, USA). RNA was isolated by using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Any leftover residual

DNA in the isolated RNA samples was removed by treating with a DNA-Free kit (Ambion, Austin, TX, USA). The pLAFR3 and pLAFR6 derivatives were conjugated into the *B. glumae* strains by triparental mating (Figurski et al., 1979).

III. Transposon mutagenesis and marker-exchange

pCSR1 was mutagenized using Tn*3-gusA* and marker-exchanged into the wild-type strain BGR1 as described previously (Bonas et al., 1989). The sites of Tn*3-gusA* insertions were determined as described previously (Kim et al., 2004). All constructs were confirmed by Southern hybridization analysis.

IV. Pellicle, swarming, and Congo red assays

The pellicle assay was performed at 28°C and 37°C for 3–4 days in LB broth in 24-well culture plates (Corning Inc., Corning, NY, USA) in static culture. The swarming assay was performed at 28°C on LB agar plates containing 0.7% agar as described previously (Kim et al., 2007). The Congo red binding assays were carried out at 28°C as described previously (Xu et al., 2013).

V. Pellicle degradation enzyme assay

Pellicles were harvested from 3- to 4-day-old cultures and washed with

Dulbecco's phosphate-buffered saline (DPBS; WELGENE, Gyeongsan, South Korea) followed by treatment with 0.86 units (U)/ml of endo-1,4-β-D-glucanase (cellulase) (Sigma-Aldrich, St. Louis, MO, USA), 0.1% proteinase K (v/v) (Sigma-Aldrich), or 0.1% RNase-free DNase I (v/v) (Qiagen, Venlo, Netherlands) as described previously (Yap et al., 2005; Liang et al., 2010). The turbidity of degraded pellicles was measured as the optical density at 600 nm (OD_{600}) using an Eppendorf BioSpectrometer kinetic (Eppendorf, Hamburg, Germany) after overnight incubation at 37°C.

VI. Analysis of c-di-GMP

C-di-GMP was extracted as described previously (Roy et al., 2013) and quantified by high-performance liquid chromatography (Dionex, Sunnyvale, CA, USA). Commercially available c-di-GMP (Sigma-Aldrich) was used as a standard.

VII. Expression of *pelI* in pSRKKm

To express a DGC gene with the PAS/PAC sensor (BGLU_RS21385) in the IPTG-inducible expression vector pSRKKm (Khan et al., 2008), the coding region was PCR amplified from BGR1 genomic DNA using the corresponding primers (BGLU_RS21385F, 5′-CATATGCTGACAACCGACACC-3′ and BGLU_RS21385R, 5′-AAGCTTTCACTCGCCGTACAGCTC-3′) with

Phusion DNA polymerase (New England Biolabs, Beverly, MA, USA). The amplified PCR product was cloned into pGEM-T Easy (Promega, Madison, WI, USA) (Table 1), followed by confirmation of correct sequences. The insert DNA was generated as a *Nde*I/*Spe*I fragment and cloned into its corresponding sites in pSRKKm. The plasmid pJW110 (Xu et al., 2013), which expresses *pleD* in pSRKKm, was introduced into the wild-type strain BGR1 as described previously (Figurski and Helinski, 1979).

VIII. Plant inoculation

The stems of rice plants (*Oryza sativa* cv. Milyang 23) were inoculated with approximately 1×10^8 colony-forming units (CFU)/ml of *B*. *glumae* strains in a plant growth chamber (Hanbaek Scientific, Bucheon, South Korea) with a 16-h light period at 30°C and an 8-h dark period at 25°C. Rice stems were photographed every 3 days after injection. The severity of disease index was calculated using the Fiji image processing software (version 1.52o; NIH) in pixels (area of diseased surface selection in square pixel × intensity of diseased area in optical density). Colonization was evaluated daily for 9 days by enumeration of recovered cells from 3 cm above and below the inoculated sites. The virulence and colonization assays were repeated three times with three independent replicates. Analysis of variance (ANOVA)/Tukey's correction for multiple comparison and significant difference at $P < 0.05$ were defined in

disease severity and colonization assays using IBM SPSS Statistics software (version 20 x86-x64; IBM, Armonk, NY, USA).

RESULTS

I. Temperature- and flagellum-dependent, but QS-independent, cellulase-sensitive pellicle formation

To determine whether temperature affects biofilm formation, cells of *B*. *glumae* were incubated in 24-well plates containing LB broth supplemented with 100 mM HEPES (pH 7.0) at 37°C and 28°C without shaking. Thin layers of pellicles appeared 3 days after inoculation of wild-type strain BGR1 at 37°C, whereas distinct pellicles were formed 2 days after inoculation at 28°C (Figure 1A). To determine the major component of pellicles produced by *B*. *glumae*, I assessed the sensitivity of pellicles to cellulase, proteinase K, and DNase I. When 4-day-old pellicles produced by wild-type strain BGR1 were treated with cellulase, the coagulated pellicles were dispersed, indicative of cellulase sensitivity, whereas no turbid dispersion was observed following treatment with 0.1% (v/v) proteinase K or 0.1% (v/v) DNase I (Figure 1B). These results indicated that a major component of pellicles is most likely composed of cellulase-sensitive materials. When 3-day-old pellicles formed at 37°C and 28°C were treated with cellulase, the turbidities were 0.301 ± 0.003 (mean ± standard deviation [SD]) and 0.681 ± 0.008, respectively (Figure 1A). These results indicated that pellicle formation was more favorable at 28°C than at the optimum growth temperature of 37°C.

Because flagellum-mediated aerotactic motility is a key factor involved in pellicle formation in aerobic bacteria, I tested whether swimming motility is critical for pellicle formation in *B*. *glumae*. Previously characterized swimming-defective mutants BGF42(BGR1, *flhA*::Tn*3-gusA42*), BGF43(BGR1, *cheB*::Tn*3-gusA43*), BGF45(BGR1, *fliA*::Tn*3-gusA45*), and BGF48(BGR1, *cheZ*::Tn*3-gusA48*) (Table 1) failed to form pellicles, and the genetically complemented strains recovered pellicle formation (Figure 2A). To determine whether QS is critical for pellicle formation in *B*. *glumae*, I performed pellicle assays with wild-type strain BGR1; two previously constructed QS-defective mutants of *B*. *glumae*, BGS2(BGR1, *tofI*::Ω) and BGS9(BGR1, *qsmR*::Ω) (Table 1); and BGS2 exogenously supplemented with 1 μM *N*-octanoyl homoserine lactone (C8-HSL). The four strains did not differ in the time required for pellicle formation or the amount of pellicle produced when grown in 24-well cell culture plates containing LB broth supplemented with 100 mM HEPES (pH 7.0) at 28°C (Figure 2B). The amount of pellicle formed in 24-well culture plates was determined through OD_{600} measurement after cellulase treatment, indicated as the mean ± SD below the respective inoculum. These results indicated that pellicle formation is independent of QS in *B*. *glumae*.

II. Identification of a gene cluster comprising putative cellulose biosynthetic genes critical for pellicle formation

Since pellicles were sensitive to cellulase, I identified genes involved in cellulose biosynthesis to determine whether cellulose biosynthesis is critical for pellicle formation. From genome information of wild-type strain BGR1, I found a gene cluster consisting of seven homologs of previously known *bcs* or regulatory genes, *bcsD* (BGLU_RS28215), *bcsR* (*yhjR*, BGLU_RS28220), *bcsQ* (*yhjQ*, BGLU_RS28225), *bcsA* (BGLU_RS28230), *bcsB* (BGLU_RS28235), *bcsZ* (*celY*, BGLU_RS28240), and *bcsC* (BGLU_RS28245) in *B*. *glumae* BGR1 (Figure 3A). The *bcs* homologs in BGR1 are indicated as black arrows in Figure 3B. Cosmid clone pCSR1, 22.03 kb in size, carrying the gene cluster identified from the previously constructed genomic library of *B*. *glumae* BGR1 was subjected to Tn*3*-*gusA* mutagenesis. Insertion of Tn*3*-*gusA* in putative cellulose biosynthetic genes was determined by direct sequencing, and each mutation was marker-exchanged into wild-type strain BGR1 to generate individual gene knockout mutants (Figure 3B). Tn*3*-*gusA* insertion in each of the seven genes abolished pellicle formation, and genetic complementation with pCSR1 *in trans* conferred recovery of phenotypes (Figure 4). Insertions in genes upstream and downstream of the seven *bcs* genes did not affect pellicle formation (Figure 3B). These results indicated that these seven genes are critical for cellulase-sensitive pellicle formation in *B*. *glumae*, despite concerns about the polar effects of Tn*3*-*gusA* insertion.

III. Constitutive expression of *pelI* and *pleD* facilitated pellicle formation and repressed swarming motility

To determine which DGC gene is the most critical for pellicle formation in *B*. *glumae*, I searched for genes encoding proteins possessing GGDEF, EAL, GGDEF/EAL, or HD-GYP motifs in the genome of *B*. *glumae* BGR1. I found 12, 7, 7, and 2 genes encoding proteins possessing GGDEF, EAL, GGDEF/EAL, and HD-GYP motifs, respectively (Table 2). Among those, I determined that a putative DGC gene (BGLU_RS21385, designated *pelI*) possessing a PAS/GGDEF motif was the most influential for pellicle formation when constitutively expressed in the multi-copy number plasmid pCOK76 in wild-type strain BGR1 (Figure 5A). Constitutive expression of *pelI* facilitated pellicle formation as assessed by measurements of turbidity following cellulase treatment of pellicles and Congo red staining, but repressed swarming motility (Figure 5A and B). In addition, constitutive expression of a heterologous gene encoding GGDEF response regulator PleD from *A*. *tumefaciens* in the multi-copy number plasmid pJW110 triggered faster and denser pellicle formation compared to the wild type carrying empty vector pSRKKm. The OD_{600} of cellulase-treated pellicles harvested 3 days after inoculation was measured, and the turbidity values of saturated pellicles of BGR1 (wild type), BGR1(pSRKKm), BGR1(pCOK76), and BGR1(pJW110) in cellulase DPBS (v/v) were 0.64 ± 0.013, 0.66 ± 0.022, 1.181 ± 0.007, and 1.179 ± 0.058,

respectively (Figure 5A). Constitutive expression of *pleD* repressed swarming motility in *B*. *glumae* (Figure 5B). These results indicated that DGC genes are critically involved in pellicle biosynthesis in *B*. *glumae*.

To confirm that constitutive expression of *pelI* and *pleD* in *B*. *glumae* affected levels of c-di-GMP, I measured the levels of c-di-GMP present in wild-type strain BGR1, BGR1 carrying *pelI* in pCOK76, and BGR1 carrying *pleD* in pJW110. C-di-GMP levels were significantly higher in BGR1 carrying pCOK76 or pJW110 than in wild-type strain BGR1 (Figure 6). These results were in good agreement with the fact that c-di-GMP is a key signal molecule for biofilm biosynthesis in bacteria.

IV. Pellicle-defective mutants were less virulent

To determine the functional role of cellulose-sensitive pellicles of *B*. *glumae* in rice plants, I inoculated pellicle-forming and non-pellicle-forming cells into stems of rice plants. The pellicle defective mutants with Tn*3-gusA* insertions in genes involved in cellulose biosynthesis caused no serious damage to rice sheaths when compared to the wild type (Figure 7A and Supplementary Figure S1A). Colonization of pellicle-defective mutants was significantly less effective than that of wild-type strain BGR1 for the 9-day period after inoculation (Figure 7B and Supplementary Figure S1B). Complementation of pellicle-defective mutants with pCSR1 carrying the seven putative cellulose

biosynthetic genes fully recovered the colonization ability and virulence (Figure 7A and B, and Supplementary Figure S1A and B). These results indicated that defects in forming cellulase-sensitive pellicles reduce the colonization ability of *B*. *glumae* in rice plants, thereby affecting virulence.

DISCUSSION

The ability to sense the available oxygen is critical for aerobes to switch their lifestyle and become sessile at the air–liquid interface and thus to form pellicles in static culture conditions (Hölscher et al., 2015; Kovács and Dragoš, 2019). While oxygen and various environmental factors are known to influence bacterial pellicle formation, little information is available as to whether temperature affects bacterial pellicle formation. Most pellicle assays are routinely performed at the optimum growth temperature of the bacterium in question. However, this does not necessarily mean that the conventional methods used for biofilm assays always apply in all cases. The physiological conditions and microenvironments of hosts when bacteria associate with their hosts should be critical for their initial interactions and colonization. In this regard, my results revealed such cases where plant pathogenic bacteria interact with their hosts. The fact that the optimal temperature for pellicle formation was different from that for growth supports the importance of temperature during interactions between plant pathogenic bacteria and their hosts. My data showed that pellicle formation is critical for the initial colonization of *B. glumae* in rice tissues, as assessed by monitoring population changes with time after inoculation. However, it was not determined whether pellicles were actually formed in rice stems by direct observation. As to mechanisms involved in the role of pellicles for colonization in *B. glumae*, I assume that cellulase-

sensitive pellicles might be helpful for initial contact and adhesion of *B*. *glumae* cells in rice tissues.

Flagellum-mediated bacterial motility is necessary for aerobes to migrate toward oxygen-rich environments in static culture conditions, but is not a requirement for pellicle development (Houry et al., 2010; Yamamoto et al., 2012; Hölscher et al., 2015). The fact that aerotactic motility accelerates pellicle formation in static culture was consistent with an observation that swimming-defective mutants fail to develop pellicles in *B*. *glumae*. Since bacterial QS has been known to play an important role in biofilm formation in gram-negative bacteria (De Kievit, 2009), it been initially hypothesized that the pellicle formation might be regulated by QS. However, QS mutants formed pellicles in buffered LB static culture conditions. The observation that pellicle formation was independent of QS was unexpected because flagellar biosynthesis is positively controlled by QS (Kim et al., 2007; Jang et al., 2014). This contradiction can be explained in two ways. First, actual flagellar biosynthesis might occur differently between flagellum-defective mutants due to null mutations in flagellar biosynthetic genes and QS-negative strains in which expression of genes involved in flagellar biosynthesis is not activated. Second, considering that bacterial pellicle formation is triggered by unfavorable environments to make adjustments for survival in gram-negative bacteria, unfavorable laboratory culture conditions such as lack of aeration and non-optimal growth temperatures might have a more crucial impact on

triggering pellicle proliferation than QS in *B*. *glumae*.

Genetic variations in *bcs* genes were grouped into type A and B (Jahn et al., 2011). A representative bacterium of type A is *Gluconacetobacter xylinus*, which is used for industrial cellulose production (Jahn et al., 2011). A single operon consisting of four genes, *bcsABCD*, is responsible for cellulose biosynthesis in *G*. *xylinus* (Jahn et al., 2011). BcsA and B are responsible for polymerization of glycan chains whereas BcsC and BcsD are involved in glucan extrusion and crystallization during cellulose assembly (Saxena et al., 1994). In type B, *bcsZ* is present between *bcsB* and *bcsC*, and there is an additional operon, *bcsEFG*, in *E*. *coli*, *Pectobacterium atrosepticum*, and *Salmonella enterica* (Le Quéré and Ghigo, 2009; Jahn et al., 2011). The genetic organization of the *bcs* gene cluster in *B*. *glumae* has unique features that do not belong to type A or B. The *bcs* gene cluster of *B*. *glumae* has the *bcsD* gene typical of type A, but also has *bcsZ* between *bcsB* and *bcsC*, and *bcsR*, unique to type B, which shows intermediate gene organization between type A and B. Such a hybrid form of *bcs* gene organization has been found in the plant pathogenic bacterium *Dickeya dadantii* (Prigent-Combaret et al., 2012). It would not be surprising to find more variations in the genetic organization of *bcs* gene clusters present in bacteria originating from diverse environmental niches.

C-di-GMP plays an important role as a second messenger in bacteria. However, a frequent encounter of difficulties in finding the most influential

genes involved in c-di-GMP biosynthesis and its degradation due to the presence of multiple copies of DGCs and PDEs was recognized (Römling and Amikam, 2006; Hengge, 2009; Morgan et al., 2014). Therefore, heterologous but well-characterized DGCs are often used to study c-di-GMP-mediated signaling in target bacteria. An initial confirmation was made in pellicle formation being controlled by c-di-GMP by adapting *pleD* from *A*. *tumefaciens*, and then identified the most critical gene, *pelI*, among 21 putative DGC genes in *B*. *glumae*. These results do not add new information to biofilm research, but it is worth finding the most influential DGC gene among many gene families in *B*. *glumae*.

Enhanced adhesion ability of rhizosphere colonizing *Pseudomonas putida* and *Pseudomonas fluorescens* via elevated levels of c-di-GMP to plant roots occurred due to facilitated biofilm formation by elevated c-di-GMP levels (Newell et al., 2009; Matilla et al., 2011). Likewise, it is likely that pellicle formation would be helpful for initial contact and adhesion of *B*. *glumae* to rice tissues, which eventually would affect the colonization ability and virulence. Nonetheless, it remains to be answered whether pellicles or biofilms produced by *B*. *glumae* truly exist around the infection sites of rice tissues. In addition to the previous reports of QS-mediated virulence mechanisms of *B*. *glumae* (Kim et al., 2004, 2007), pellicle was found to be an important virulence factor independent of QS. This finding may provide a new drug target to control rice panicle blight caused by *B*. *glumae*

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Table 1. Strains and plasmids used in this study.

Bacterial strain or plasmid	Genotype[a]	Source or reference
Strains		
Burkholderia glumae		
BGR1	Wild-type, Rif ^{R}	Jeong et al. (2003)
BGS2	BGR1, *tofI*::Ω	Kim et al. (2004)
BGS9	BGR1, *qsmR*::Ω	Kim et al. (2007)
BGA90	BGR1, *bcsA*::Tn*3-gusA90*	This study
BGB107	BGR1, *bcsB*::Tn*3-gusA107*	This study
BGC45	BGR1, *bcsC*::Tn*3-gusA45*	This study
BGD67	BGR1, *bcsD*::Tn*3-gusA67*	This study
BGZ193	BGR1, *bcsZ*::Tn*3-gusA193*	This study

Continued on the following page

Table 1 – *Continued*

BGQ6	BGR1, *bcsQ*::Tn*3-gusA6*	This study
BGR88	BGR1, *bcsR*::Tn*3-gusA88*	This study
BGF42	BGR1, *flhA*::Tn*3-gusA42*	Kim et al. (2007)
BGF43	BGR1, *cheB*::Tn*3-gusA43*	Kim et al. (2007)
BGF45	BGR1, *fliA*::Tn*3-gusA45*	Kim et al. (2007)
BGF48	BGR1, *cheZ*::Tn*3-gusA48*	Kim et al. (2007)
Escherichia coli		
DH5α	F^- Φ80d*lacZ*Δ*M15*Δ(*lacZYA-argF*) *U169* endA1 *recA1* *hsd1hsdR17*($r_k^-m_k^+$) *deoRthi-1supE44*λ^- *gyrA96 relA1*	Gibco BRL

Continued on the following page

Table 1 – *Continued*

S17-1	Tra^{+}, *recA*, Sp^{R}	Simon et al. (1983)
C2110	*polA*, Nal^{R}	Stachel et al. (1985)
Plasmids		
pSRKKm	pBBR1MCS-2-derived broad-host-range expression vector containing lac promoter and lacI^{q}, lacZα^{+}, and Km^{R}	Khan et al. (2008)
pBluescript IISK(+)	Cloning vehicle; phagemid, pUC derivative, Amp^{R}	Stratagene
pLysS	Encodes T7 lysozyme gene, Cm^{R}	Novagen
pSShe	Cm^{R}	Stachel et al. (1985)
pHoKmGus	Promoterless *β*-glucuronidase gene; Km^{R} Amp^{R} *tnpA*	Bonas et al. (1989)

Continued on the following page

Table 1 – *Continued*

pLAFR3	Tra^{-}, Mob^{+}, RK2 replicon, Tet^{R}	Staskawicz et al. (1987)
pJW110	pSRKKm carrying P_{lac}-*pleD*; Km^{R}	Xu et al. (2013)
pCOK76	Plasmid vector carrying *lacI*^{q}, P*lac*, *lacZα*, and *pelI*, Gm^{R}	This study
pCSR1	Plasmid harboring *bcsA, B, C, D, Z,* and *yhjQ*	This study
pBGF2	23 kb DNA fragment harboring *fliA*, *flhA*, *CheZ*, and *CheB* from BGR1 cloned into pLAFR3	Kim et al. (2007)

[a] Amp^{R}, ampcilin resistance; Cm^{R}, chloramphenicol resistance; Km^{R}, Kanamycin resistance; Nal^{R}, nalidixic acid resistance; Rif^{R}, rifampicin resistance; Sp^{R}, spectinomycin resistance; Tet^{R}, tetracycline resistance.

Table 2. Identified GGDEF/EAL domain encoding genes in *B. glumae.*

Locus ID*	Domain	Annotation
BGLU_RS01045	REC/GGEEF	Diguanylate cyclase6
BGLU_RS05070	PAC/GGDEF/EAL	Diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s)
BGLU_RS05175	EAL	Hypothetical protein BDAG_01993
BGLU_RS06570	GGDEF/EAL	Diguanylate cyclase/phosphodiesterase with PAS/PAC sensor (frame shifted)
BGLU_RS09190	GGDEF/EAL	Diguanylate cyclase3
BGLU_RS12055	EAL/HDOD	Diguanylate phosphodiesterase2/*Burkholderia cenocepacia* pBCA055

Continued on the following page

Table 2 – *Continued*

BGLU_RS13960	GGDEF/EAL	Hypothetical protein BMASAVP1 A1055
BGLU_RS14170	GGDEF/EAL	Diguanylate cyclase/phophodiesterase2
BGLU_RS17000	EAL	Cyclic diguanylate phosphodiesterase
BGLU_RS17085	GGDEF/EAL	Diguanylate cyclase/phosphodiesterase
BGLU_RS17210	GGDEF	Porin, Gram-negative type
BGLU_RS18985	GGDEF	Hypothetical protein BB4664
BGLU_RS21375	GGDEF/EAL	Glycogen debranching enzyme Glgx4
BGLU_RS21385	PAS/GGDEF	Diguanylate cyclase with PAS/PAC sensor (*pelI*)

Continued on the following page

Table 2 – *Continued*

BGLU_RS22710	GGDEF/EAL	Thioester reductase
BGLU_RS25010	GGDEF/EAL	Diguanylate cyclase/phophodiesterase4
BGLU_RS25390	GGDEF	Glycogen debranching enzyme Glgx2
BGLU_RS27365	EAL	Diguanylate phosphodiesterase4
BGLU_RS28165	EAL	Diguanylate phosphodiesterase3
BGLU_RS28585	GGDEF/EAL	Diguanylate cyclase/phophodiesterase3

*Locus IDs were obtained from GenBank.

Figure 1. Temperature-dependent pellicle formation in *Burkholderia glumae* and enzymatic pellicle degradation. (A) Pellicle formation was observed for 3 days at 37°C and 28°C. A distinctive pellicle of the wild-type strain BGR1 was observed after 3 days of incubation and was more evident at 28°C than at 37°C. The turbidity of cellulase-treated pellicles from the respective temperatures was assessed on the 3rd day of incubation and is shown as the mean ± standard deviation. (B) The pellicle harvested from the static culture of wild-type strain BGR1 at 28°C was treated with cellulase, DNase I, or proteinase K in Dulbecco's phosphate-buffered saline. The pellicle was degraded by the addition of cellulase, but DNase I and proteinase K did not degrade the pellicle.

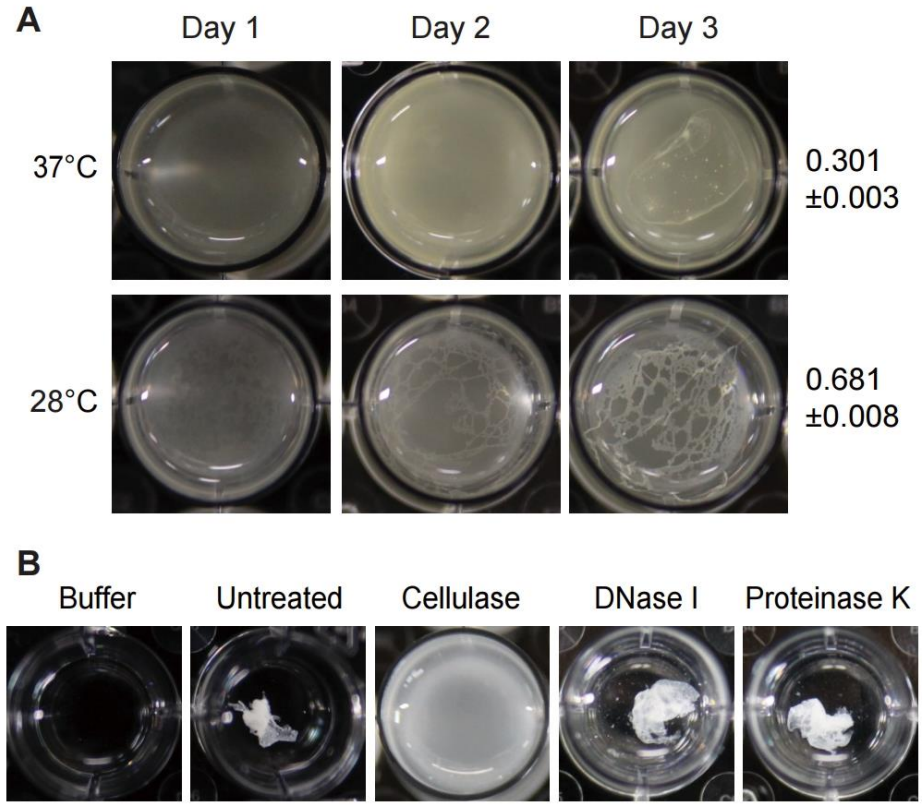

A
Day 1
Day 2
Day 3
37°C
0.301
±0.003
28°C
0.681
±0.008
B
Buffer
Untreated
Cellulase
DNase I
Proteinase K

Figure 2. Flagella-dependent but quorum sensing-independent pellicle formation in *B*. *glumae*. (A) Pellicle formation of wild type, swimming-defective mutants, and complemented strains with pBGF2. All swimming-defective mutants, BGF42(BGR1, *flhA*::Tn*3-gusA42*), BGF43(BGR1, *cheB*::Tn*3-gusA43*), BGF45(BGR1, *fliA*::Tn*3-gusA45*), and BGF48(BGR1, *cheZ*::Tn*3-gusA48*) exhibited no pellicle formation. Genetic complementation of swimming-defective mutants with pBGF2 carrying a cluster of flagellar genes restored pellicle deficiencies in all swimming-defective mutants used. (B) Pellicle formation of wild-type BGR1, QS mutant BGS2(BGR1, *tofI*::Ω), BGS2 with exogenously added 1 μM C8-HSL, and *qsmR* mutant BGS9(BGR1, *qsmR*::Ω) in Luria-Bertani broth buffered with 100 mM HEPES (pH 7.0) under static culture at 28°C. Turbidity measurements at 600 nm following cellulase treatment are shown as the mean ± SD.

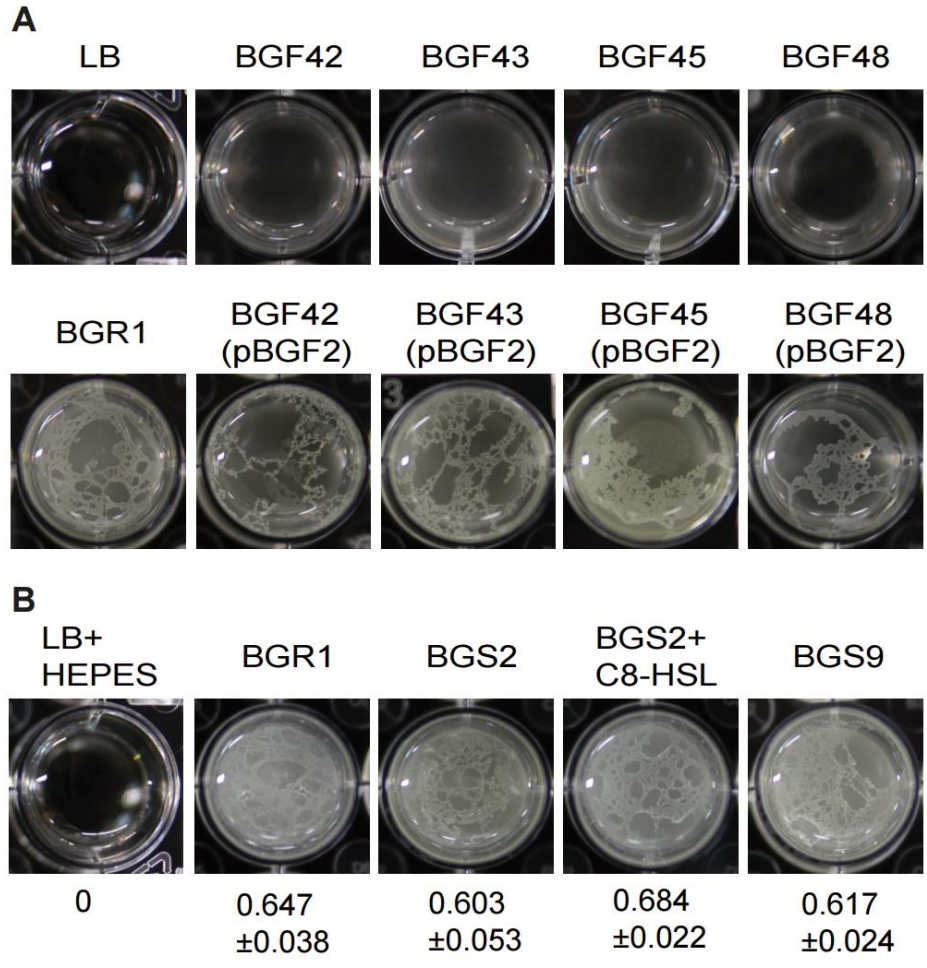

A
LB
BGF42
BGF43
BGF45
BGF48
BGR1
BGF42 (pBGF2)
BGF43 (pBGF2)
BGF45 (pBGF2)
BGF48 (pBGF2)
B
LB+ HEPES
BGR1
BGS2
BGS2+ C8-HSL
BGS9
0
0.647 ±0.038
0.603 ±0.053
0.684 ±0.022
0.617 ±0.024

Figure 3. Physical maps of bacterial cellulose biosynthetic genes. (A) Type operons of the cellulose biosynthetic genes of pellicle-producing bacteria: type A, *G. xylinus* ATCC23769 (Saxena et al., 1994); type B, *E. coli* K-12 (Le Quéré and Ghigo, 2009); and hybrid type AB, *D. dadantii* 3937 (Jahn et al., 2011). Homologous genes are placed in vertical alignment. Open reading frames (ORFs) are drawn to scale. (B) Genetic map of plasmid pCSR1 including cellulose biosynthetic genes of *B. glumae* BGR1. The Tn*3-gusA* insertion and its pellicle formation (−/+) are marked below the map. B, *Bam*HI; E, *Eco*RI; and S, *Sac*I. The enzyme sites in parentheses, (B) and (E), are the enzyme sites in pLAFR3 (Table 1). The ORFs are drawn to scale. The scale bars in (A) and (B) represent 1 kb.

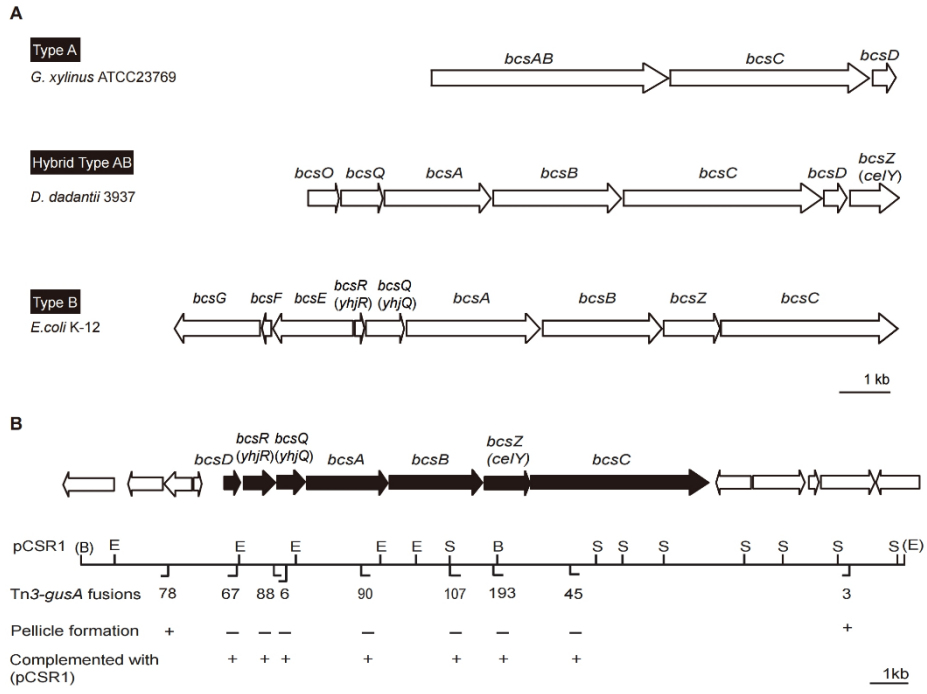

A
Type A
G. xylinus ATCC23769
bcsAB
bcsC
bcsD
Hybrid Type AB
D. dadantii 3937
bcsO
bcsQ
bcsA
bcsB
bcsC
bcsD
bcsZ (celY)
Type B
E.coli K-12
bcsG
bcsF
bcsE
bcsR (yhjR)
bcsQ (yhjQ)
bcsA
bcsB
bcsZ
bcsC
1 kb
B
bcsD
bcsR (yhjR)
bcsQ (yhjQ)
bcsA
bcsB
bcsZ (celY)
bcsC
pCSR1 (B) E E E E E S B S S S S S S (E)
Tn3-gusA fusions 78 67 88 6 90 107 193 45 3
Pellicle formation + – – – – – – – +
Complemented with (pCSR1) + + + + + + +
1kb

Figure 4. Pellicle formation of cellulose mutants and complementation strains. All cellulose mutants abolished pellicle formation and their pellicle deficiencies were recovered by complementation with plasmid pCSR1 harboring a cluster of cellulose biosynthesis genes.

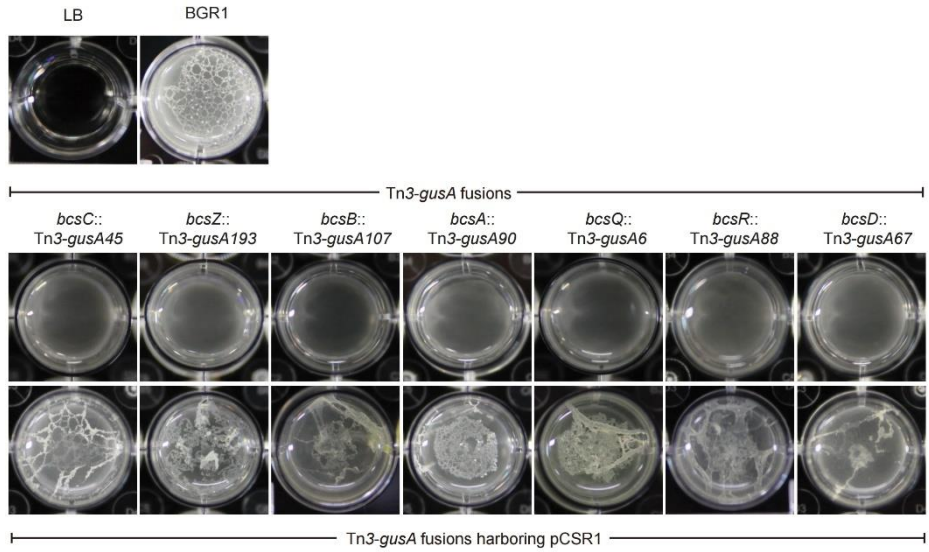

LB
BGR1
Tn3-gusA fusions
bcsC:: Tn3-gusA45
bcsZ:: Tn3-gusA193
bcsB:: Tn3-gusA107
bcsA:: Tn3-gusA90
bcsQ:: Tn3-gusA6
bcsR:: Tn3-gusA88
bcsD:: Tn3-gusA67
Tn3-gusA fusions harboring pCSR1

Figure 5. Pellicle formation, swarming motility, and Congo red binding assay. (A) Pellicle formation of wild-type strain BGR1, BGR1(pSRKKm, empty vector), BGR1(pCOK76; pSRKKm::*pelI*), and BGR1(pJW110; pSRKKm::*pleD*). After 3 days of incubation, pellicles were harvested, treated with cellulase, and the turbidity was measured at 600 nm. The asterisks (*) represent a significant difference ($P < 0.05$) in turbidity between wild-type strain BGR1, BGR1(pCOK76), and BGR1(pJW110). (B) Swarming motility and Congo red binding activity of wild type BGR1, BGR1(pCOK76; pSRKKm:*pelI*), and BGR1(pJW110; pSRKKm::*pleD*).

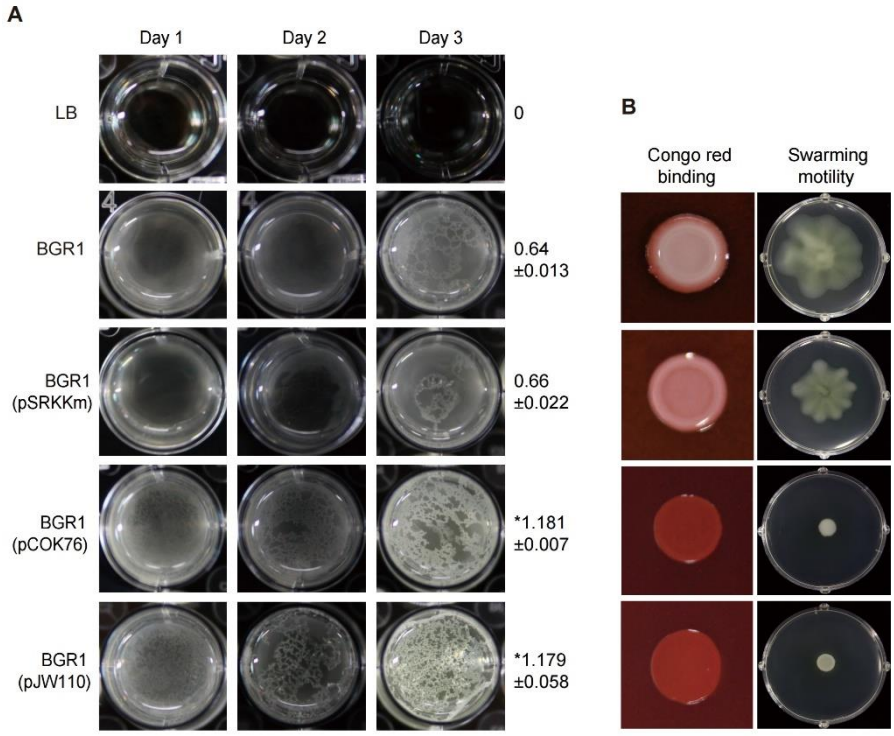

A
Day 1
Day 2
Day 3
LB
0
BGR1
0.64
±0.013
BGR1
(pSRKKm)
0.66
±0.022
BGR1
(pCOK76)
*1.181
±0.007
BGR1
(pJW110)
*1.179
±0.058
B
Congo red
binding
Swarming
motility

Figure 6. Quantification of c-di-GMP in wild-type strain BGR1, BGR1(pCOK76; pSRKKm::*pelI*), and BGR1(pJW110; pSRKKm::*pleD*) using LC/MS analysis. BGR1(pCOK76; pSRKKm::*pelI*) and BGR1(pJW110; pSRKKm::*pleD*) strains exhibited increased production of c-di-GMP. The asterisks (*) represent a significant difference ($P < 0.05$) in c-di-GMP among wild-type strain BGR1, BGR1(pCOK76), and BGR1(pJW110).

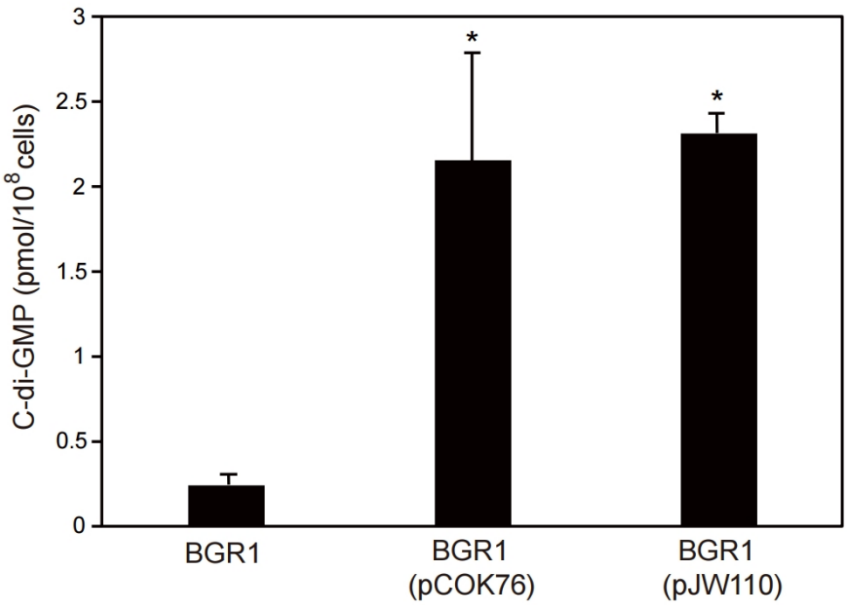

C-di-GMP (pmol/10^8 cells)
3
2.5
2
1.5
1
0.5
0
*
*
BGR1
BGR1
(pCOK76)
BGR1
(pJW110)

Figure 7. Virulence of wild type, non-pellicle producing cellulose mutants, and complementation strains in rice sheath. (A) The pellicle-defective mutants with Tn*3*-*gusA* insertion in putative cellulose biosynthetic genes, *bcsQ* and *bcsC*, exhibited no serious symptoms in rice sheaths compared to the wild type, with a score value of 141.85 ± 12.34. The virulence of non-pellicle producing cellulose mutants was recovered by complementation with pCSR1. The diseased areas in wild type, non-pellicle producing cellulose mutants, and complemented strains were scored in pixels with SDs. The superscripts (a, b, and c) before the mean values indicate significant differences ($P < 0.05$) based on ANOVA/Tukey's correction for multiple comparisons. (B) Changes in population of wild type, non-pellicle producing cellulose mutants, and complementation strains in the inoculated sheath. Non-pellicle producing cellulose mutants exhibited significant differences compared to the wild type. Population density in non-pellicle producing cellulose mutants was recovered by complementation with *bcs* operon-carrying pCSR1. The error bars represent three independent inoculation experiments. Two representative data are shown in this figure; the others are shown in Figure 8.

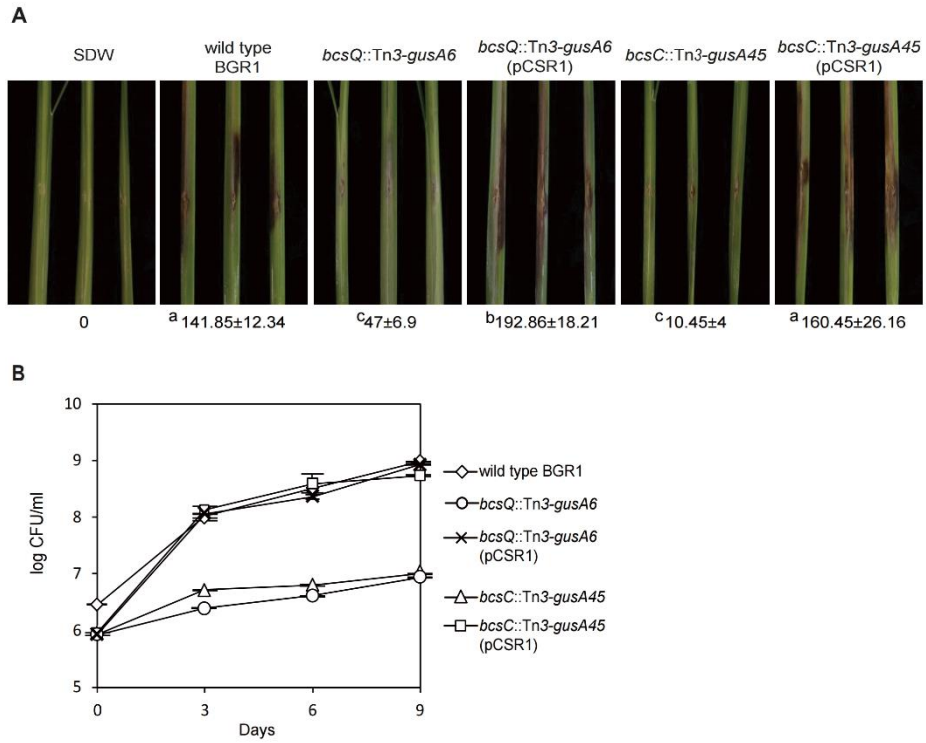
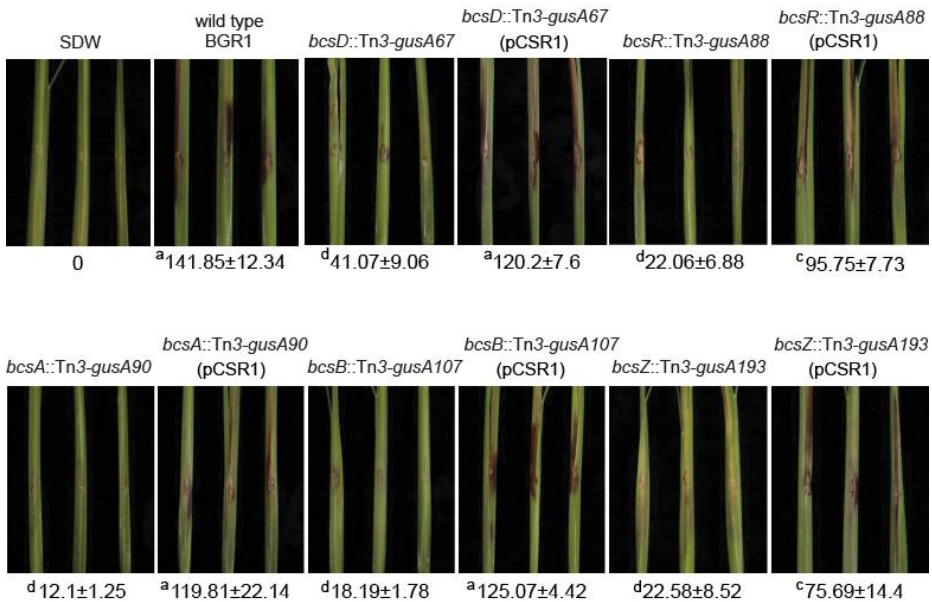
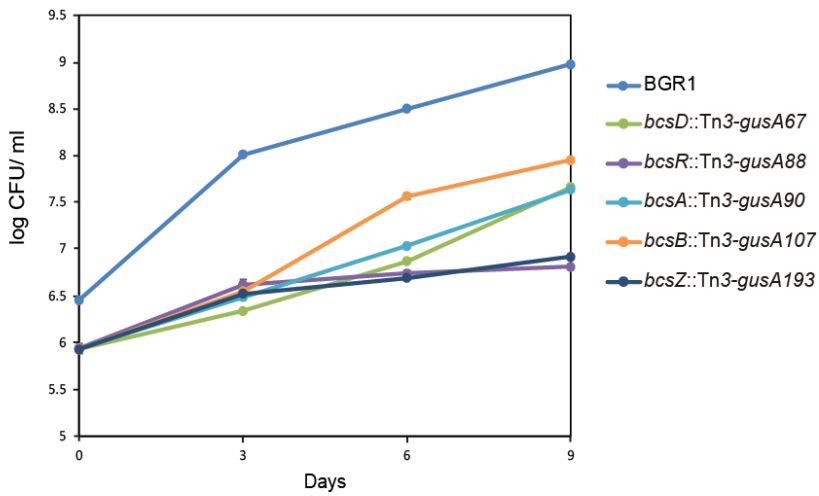

A
SDW
wild type BGR1
bcsQ::Tn3-gusA6
bcsQ::Tn3-gusA6 (pCSR1)
bcsC::Tn3-gusA45
bcsC::Tn3-gusA45 (pCSR1)
0
a 141.85±12.34
c 47±6.9
b 192.86±18.21
c 10.45±4
a 160.45±26.16
B
log CFU/ml
Days
wild type BGR1
bcsQ::Tn3-gusA6
bcsQ::Tn3-gusA6 (pCSR1)
bcsC::Tn3-gusA45
bcsC::Tn3-gusA45 (pCSR1)

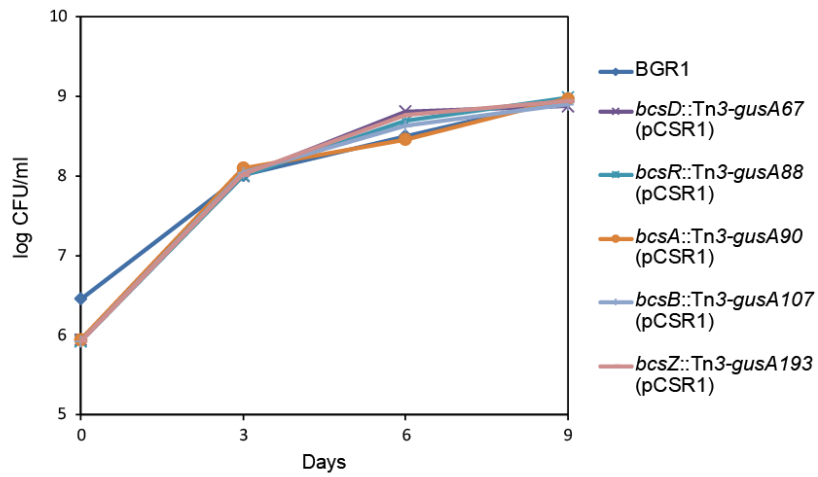
Figure 8. Virulence assay of non-pellicle forming cellulose mutant strains. (A) The pellicle defective mutants with Tn*3*-*gusA* insertion in putative cellulose biosynthetic genes, *bcsD*, *R*, *A*, *B*, and *Z*, exhibited no serious symptoms in rice sheaths compared to wild-type strain BGR1. The complemented strains exhibited full recovery of virulence. The superscripts (a, b, c, and d) on the mean values represent a significant difference ($p < 0.05$) based on ANOVA/Tukey's correction for multiple comparisons in virulence scores in pixels among treatments. Values are presented as the mean ± standard deviation. (B) Changes in population of wild type, non-pellicle producing cellulose mutants, and complementation strains in rice sheaths. The colonization ability of the cellulose mutants with restored pellicle production was recovered to the level of wild-type strain BGR1.

A


SDW
wild type BGR1
bcsD::Tn3-gusA67
bcsD::Tn3-gusA67 (pCSR1)
bcsR::Tn3-gusA88
bcsR::Tn3-gusA88 (pCSR1)
0
a141.85±12.34
d41.07±9.06
a120.2±7.6
d22.06±6.88
c95.75±7.73
bcsA::Tn3-gusA90
bcsA::Tn3-gusA90 (pCSR1)
bcsB::Tn3-gusA107
bcsB::Tn3-gusA107 (pCSR1)
bcsZ::Tn3-gusA193
bcsZ::Tn3-gusA193 (pCSR1)
d12.1±1.25
a119.81±22.14
d18.19±1.78
a125.07±4.42
d22.58±8.52
c75.69±14.4

B


log CFU/ ml
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
0
3
6
9
Days
BGR1
bcsD::Tn3-gusA67
bcsR::Tn3-gusA88
bcsA::Tn3-gusA90
bcsB::Tn3-gusA107
bcsZ::Tn3-gusA193


log CFU/ml
10
9
8
7
6
5
0
3
6
9
Days
BGR1
bcsD::Tn3-gusA67 (pCSR1)
bcsR::Tn3-gusA88 (pCSR1)
bcsA::Tn3-gusA90 (pCSR1)
bcsB::Tn3-gusA107 (pCSR1)
bcsZ::Tn3-gusA193 (pCSR1)

CHAPTER 2

Adverse effects of adaptive mutation to survive static culture conditions on successful fitness of the rice pathogen *Burkholderia glumae* in a host

ABSTRACT

Bacteria often possess relatively flexible genome structures and adaptive genetic variants that allow survival in unfavorable growth conditions. Bacterial survival tactics in disadvantageous microenvironments include mutations that are beneficial against threats in their niche. Here, a report of the aerobic rice bacterial pathogen *Burkholderia glumae* BGR1 changes a specific gene for improved survival in static culture conditions is made. Static culture triggered formation of colony variants with deletions or point mutations in the gene *bspP* (BGLU_RS28885), which putatively encodes a protein that contains PDC2, PAS-9, SpoIIE, and HATPase domains. The null mutant of *bspP* survived longer in static culture conditions and produced a higher level of bis-(3′-5′)-cyclic dimeric guanosine monophosphate than the wild type. Expression of the bacterial cellulose synthase regulator (*bcsB*) gene was upregulated in the mutant, consistent with the observation that the mutant formed pellicles faster than the wild type. Mature pellicle formation was observed in the *bspP* mutant before pellicle formation in wild-type BGR1. However, the population density of the *bspP* null mutant decreased substantially when grown in Luria–Bertani medium with vigorous agitation due to failure of oxalate-mediated detoxification of the alkaline environment. The *bspP* null mutant was less virulent and exhibited less effective colonization of rice plants than the wild type. All

phenotypes caused by mutations in *bspP* were recovered to those of the wild type by genetic complementation. Thus, although wild-type *B*. *glumae* BGR1 prolonged viability by spontaneous mutation under static culture conditions, such genetic changes negatively affected colonization in rice plants. These results suggest that adaptive gene sacrifice of *B*. *glumae* to survive unfavorable growth conditions is not always desirable as it can adversely affect adaptability in the host.

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Kwak G-Y., Goo E., Jeong H., Hwang I. (2020). Adverse effects of adaptive mutation to survive static culture conditions on successful fitness of the rice pathogen *Burkholderia glumae* in a host. *PLoS ONE*, 15: e0238151.

INTRODUCTION

Bacterial genomes are flexible and can be easily changed under different growth conditions for survival (Francino et al., 2012). Bacteria often acquire beneficial genetic traits by mutations to adapt to their environment (Francino et al., 2012). Although bacterial adaptation to variable environmental conditions is essential, it is not always possible to track such changes easily. Thus, experimental evolution of bacteria is useful for the analysis of genetic changes that occur in a relatively short period of time and can improve understanding of the bacterial response to environmental cues in nature (Francino et al., 2012; Giri et al., 2019, Rainey et al., 1998).

The occurrence of mutations under specific bacterial growth conditions, such as bacterial biofilm microcosms, is considered a competitive and defensive response for survival (Rainey et al., 2017; Martin et al., 2016). Synthesized bacterial cellulosic biofilms float at the air–liquid interface as pellicles where available oxygen is most abundant, and cells with various colony forms exist at the interface (Rainey et al., 2017; Steenackers et al., 2016; Spiers et al.,2003). Spatially constrained environments, such as static culture conditions in which sufficient oxygen for normal growth of aerobic bacteria is lacking, require aerobic bacteria to survive and adapt in an oxygen-deficient environment (Spiers et al., 2003; Morris et al., 2014; Lind et

al., 2015). This forces them to evolve in ways that maximize their metabolism for survival (D'Souza et al. 2014; Koskiniemi et al., 2012; Frenoy et al., 2018).

Considering that aerobic plant pathogenic bacteria infect plant tissues and proliferate in the intercellular space, it is worth noting the physiological conditions of the apoplast. While it is known that the intercellular space of plant cells is filled with air, the reason for this is unclear (Sattelmacher et al., 2007; Sattelmacher et al., 2001;Woolley et al., 1983;Gentzel et al., 2019). The environment of the apoplast, however, is quite different from the artificial shaking culture that supplies sufficient air and nutrients. Therefore, it would be interesting to understand the survival strategies of plant pathogenic bacteria in oxygen-limited conditions in contrast to artificial shaking culture. The rice bacterial pathogen *Burkholderia glumae* BGR1 was used as a model organism and investigated how it genetically and physiologically adapts to oxygen-limited conditions. As an aerobic bacterium, *B. glumae* causes rice panicle blight that can result in serious economic damage when weather conditions are favorable for infection and colonization (Kim et al., 2004; Kim et al., 2007).

This bacterium uses amino acids as carbon and nitrogen sources in culture media such as LB (Goo et al., 2012). Use of amino acids as carbon sources releases ammonia as a result of deamination, which causes an alkaline environmental pH (Goo et al., 2017). High alkalinity is toxic to *B. glumae*; the wild-type strain BGR1 produces the strong acidic compound oxalate to detoxify the alkalinity in a quorum

sensing (QS) manner (Goo et al., 2017). Such detoxification is essential for the survival of *B*. *glumae* when grown in amino acid-rich media (Goo et al., 2012). A previous report regarding the *B*. *glumae* producing cellulase-sensitive pellicles that is dependent on bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) but independent of QS in static culture (Kwak et al., 2020), which led to an investigation in determining how the cells of *B*. *glumae* behave physiologically and genetically in a pellicle-forming environment.

In this study, three specific questions regarding the survival and adaptation of *B*. *glumae* in static culture conditions are addressed. First, a determination of survival behaviors of *B*. *glumae* in static culture compared to shaking culture. Second, a determination of whether the *B*. *glumae* undergoes genetic changes to improve survival in static culture. Finally, characterization of colony variants was found to increase the survival rates in static culture. The static culture condition was found to trigger genetic changes in the gene *bspP* (BGLU_RS28885), possibly encoding a protein of 108 kDa. Such mutants showed abnormal colony morphologies, that varies from the wild-type smooth BGR1 colony morphology, formed pellicles faster, and exhibited improved survival compared to the wild type in static culture but failed to colonize successfully in the host. These results suggest that adaptive evolution by sacrifice of a specific gene to overcome unfavorable growth conditions could cause unexpected and devastating outcomes for plant pathogenic bacteria such as *B*. *glumae*.

MATERIALS AND METHODS

I. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. glumae* and *Escherichia coli* were aerobically grown at 250 rpm and 37°C in LB broth (0.1% tryptone, 0.5% yeast extract, and 0.5% NaCl; USB Corp.) with the following antibiotics: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; trimethoprim, 75 μg/ml; rifampicin, 100 μg/ml; and spectinomycin, 100 μg/ml. LB agar medium contains 1.5% (w/v) agar (BD Biosciences). *B. glumae* strains were grown at 28°C and 37°C in liquid LB in 24-well plates (Corning, Inc.) as static culture. Aliquots of 100 μl from 24-well plates were serially diluted and spread on LB agar medium on day 0 through 7. The harvested samples were taken from different wells of 24- well plate to minimize possible interference involved in daily sampling for monitoring colony forming unit (CFU) colony morphology. Hence, the 7 wells were inoculated with the same sample for each day's sampling for a week. The plates were incubated at 37°C for 24 h to allow colonies to develop and at room temperature up to 2 days. Colony variants were isolated on day 3 of incubation at 28°C.

II. Pellicle assays

Cells were inoculated in 2 ml LB broth with appropriate antibiotics and grown at 37°C at 250 rpm for 18 h. Overnight cultures were washed twice with fresh LB broth, and turbidity was adjusted to an optical density of 0.05 at 600 nm using an Eppendorf BioSpectrometer kinetic (Eppendorf) followed by subculture in 24-well plates (Corning, Inc.). Pellicles were formed and harvested from the 3rd day of incubation period.

III. Nucleic acid extraction and manipulation

Standard DNA cloning, restriction mapping, and gel electrophoresis methods were used (Sambrook et al., 1989). Vector plasmid DNA and genomic DNA were treated with appropriate restriction enzymes as recommended by the suppliers (New England Biolabs and TaKaRa). Extraction of DNA fragments from agarose gels was performed as described by the manufacturer (Qiagen). A previously constructed cosmid genomic library was used (Kim et al., 2004). The pLAFR3 and pLAFR6 derivatives were mobilized into *B. glumae* strains by triparental mating (Figurski et al., 1979). Plasmids were extracted with the QIAGEN® Plasmid Mini Kit (Qiagen) according to the manufacturer's instructions. For purification of PCR products, agarose gel and vector DNA, QIAEX II® Gel Extraction Kit (Qiagen), and BIONEER *AccuPrep*® PCR Purification Kit (Bioneer) were used according to the manufacturers' instructions. The target gene was amplified via PCR using a thermocycler (Model C1000; Bio-Rad Laboratories)

with Phusion DNA polymerase (New England Biolabs).

IV. Mutagenesis of *bspP*

The mutagenized plasmid carrying a Tn*3*-*gusA* insertion was introduced into the parent strain by bacterial conjugation followed by marker exchange as previously described (Bonas et al., 1989). The insertion site and orientation of Tn*3*-*gusA* in the mutant were determined by restriction enzyme digestion and direct sequencing of the plasmid using the primer Tn3-gusA (5'-CCGGTCATCTGAGACCATTAAAAGA-3') as previously described (Kim et al., 2004). EZ-Tn*5* insertional mutagenesis of pPAS1 for genetic complementation of the *bspP* null mutant was carried out using the EZ-Tn*5*^{TM} <DHFR-1> Tnp Transposome^{TM} Kit (Epicentre Technologies, Corp.) according to the manufacturer's instructions. The insertion site was determined using the primers provided in the kit. Marker exchange was confirmed by Southern hybridization analyses. The assembly and analysis of the CS-type 3-2A mutant strain were carried out in the following manner: Illumina shotgun libraries from gDNA were prepared and sequenced on one lane of an Illumina HiSeq 2500 sequencer. Resequenced Illumina HiSeq data were trimmed using Trimmomatic software and aligned to the *B. glumae* BGR1 genome using the BWA package. DNA variants, including single nucleotide polymorphisms, insertions, and deletions, between libraries were detected using SAMtools and FreeBayes (Goo et al., 2017).

V. Oxalate and QS signal assays

B. glumae was grown in LB or LB supplemented with 100 mM 4-(2-hydroxyethyl) piperazin-1 ethanesulfonic acid (HEPES) to assess oxalate production and QS signals. An oxalate assay kit (Libios) was used according to the manufacturer's instructions and as described previously (Goo et al., 2017; Laker et al., 1980). An autoinducer assay was performed as described previously described (Kim et al., 2004) with the following modification: wild-type BGR1 and *bspP* null mutant BGP38 were grown in LB supplemented with HEPES (pH 7.0) for 1 day.

VI. Quantification of pellicles by cellulase treatment

The harvested 3- to 4-day-old pellicles were washed with Dulbecco's phosphate-buffered saline (Welgene) followed by treatment with 0.86 units (U)/ml 1,4-(1,3:1,4)-β-D-glucan 4-glucanohydrolase (v/v) (Calbiochem-Novabiochem), 0.1% proteinase K (v/v) (Sigma-Aldrich), or 0.1% RNase-free DNase I (v/v) (Qiagen) as described previously (Liang et al., 2010; Yap et al., 2550). After overnight incubation at 37°C, the turbidity of the degraded pellicles was measured as the optical density at 600 nm (OD_{600}) using an Eppendorf BioSpectrometer kinetic.

VII. Analyses of c-di-GMP

The extraction of c-di-GMP was performed as described previously (Roy et al., 2013) and quantification of extracted c-di-GMP was carried out by high performance liquid chromatography (Dionex). Commercially available c-di-GMP (Sigma-Aldrich) was used as a reference.

VIII. Quantitative real-time polymerase chain reaction (qRT-PCR)

Isolation of total RNA from wild-type BGR1, *bspP* null mutant BGP38, and the *bspP* complementation strain BGP38C was performed using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNaseI treatment of the isolated RNA, reverse transcription to cDNA, and RT-PCR were performed as described previously (Jang et al., 2014). qRT-PCR was performed as follows: 95°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and 72°C for 5 min using a thermocycler (Model C1000; Bio-Rad Laboratories). The primers used for qRT-PCR are listed in Table 2.

IX. Statistical analysis

Results from the oxalate assay, cellulase assay, and c-di-GMP analysis were compared using analysis of variance with Tukey correction for multiple comparisons. A P-value <0.05 was taken to denote statistical significance. The normalized fold expression was calculated for *bcsB* and *obcA*. All analyses were

performed on data from three biological replicates using IBM SPSS Statistics software (version 25.0; IBM Corporation).

X. Plant inoculation

Virulence and colonization assays of *B. glumae* strains in *Oryza sativa* cv. Milyang 23 were performed as described previously (Kwak et al., 2020). Data were obtained from three biological replicates.

RESULTS

I. Emergence of morphologically distinct spontaneous mutants in static culture

To determine the viability of aerobic *B*. *glumae* wild-type BGR1 in static culture, aliquots of the static culture were taken each day for a week. Cell densities increased and reached 1×10^9 colony forming units (CFU)/ml 2 days after subculture in 24-well plates (Fig. 1A). However, two types of abnormal colony variants distinctively different from the wild-type BGR1 colonies appeared 3 days after incubation; one had a crater-like shape in the middle of the colony (CS-type) and the other had an irregular boundary (IR-type) (Fig. 1B). Combined numbers of both types of colony variants continued to increase exponentially, reaching approximately 90% of whole live cells while the number of total viable cells was maintained at approximately 1×10^9 CFU after 7 days (Fig. 1A). On the 3rd day to 7th day of the static culture incubation period, CS-type colonies occupied 94%, 87%, 80%, 86%, and 88% of dominant occupancy over the IR-type colonies respectively.

II. Identification of *bspP* mutations in spontaneous mutants

To determine whether genetic mutations caused colony morphological

changes, whole genome sequencing of the CS-type 3–2A mutant was obtained. The identified point mutation was at the 3′ end of the annotated gene (BGLU_RS28885) called *bspP* (Fig. 2). The *bspP* gene was 3042 bp and putatively encodes a protein of 108 kDa with four known domains: second PDC (Pho/Dus/CitA) sensor domain of diguanylate-cyclase (PDC2), signal transduction mechanism domain (PAS-9), stage II sporulation protein E (SpoIIE) domain, and histidine kinase-like ATPase (HATPase) domain (Fig. 2).

In the 3–2A mutant, nucleotide 3024 (A) in *bspP* was changed to T (denoted as 3024A→T), changing isoleucine to phenylalanine (denoted as I984F) in BspP (Fig. 2). To determine whether similar mutations found in the 3–2A mutant occurred in *bspP* in other mutants, the *bspP* gene was amplified in other mutants with the primers BGLU_RS28885F and BGLU_RS28885R Table 2) followed by direct sequencing of the coding region of *bspP*. The various mutations in *bspP* were identified to be in the spontaneously occurring mutants BGA1, BGS8, R1S1, R1S2, R1S3, R1S4, R1S10, and BGA2 (Fig. 2). There was a 95 bp deletion at the 5′ end of *bspP* in BGA1, and 132 bp and 917 bp deletions at the 3′ ends of *bspP* in R1S1 and BGS8, respectively (Fig. 2). One and two base pair deletions were found near the 3′ ends of *bspP* in R1S10 and R1S3, respectively, (Fig. 2). Single point mutations, such as 2791A→G (N926S) and 3012G→A (G980S), were found in R1S2 and R1S4, respectively (Fig. 2). Several point mutations (7C→G, 29A→G, 34A→G, 48C→T, 70G→A, and 82G→A) were identified in BGA2, causing the

following amino acid changes, respectively: R3G, H10R, T12A, A17A (no amino acid changed due to 48C→T), V24M, and G28R (Fig. 2). 3–2A, R1S1, R1S2, R1S3, R1S4, and R1S10 exhibited mutations in the HATPase domain, and BGS8 in the SpoIIE domain of BspP (Fig. 2). As a result of the mutations in *bspP*, BGA1 and BGS8 underwent premature termination (Fig. 2). To confirm that a mutation in *bspP* confers morphological changes in *B*. *glumae*, pPAS1 carrying *bspP* was mutagenized with Tn*3-gusA* followed by marker exchange into wild-type BGR1. The resulting mutant BGP38 (BGR1 *bspP*::Tn*3-gusA38*) showed CS-type colony morphology as observed in 3–2A, R1S1, R1S2, R1S3, R1S4, R1S10, and BGA2 (Fig. 2). Two other plant pathogenic *Burkholderia* species, *B*. *plantari* and *B*. *gladioli*, possess a homolog of BspP with 94.08% and 84.42% identity at the amino acid level, respectively. Another plant pathogen, *Ralstonia solanacearum*, had a BspP homolog with 51.74% homology (Fig. 3). Homologous PDC2, SpoIIE, and HATPas domains were also found in *B*. *plantari*, *B*. *gladioli*, and *R*. *solanacearum*. Heme binding pockets were also present in all four strains with the exception that the sensory-box was present in *B*. *gladioli* and *R*. *solanacearum* (Fig. 3). The presence of the PAS-9 domain was an exception, and was found only in *R*. *solanacearum* (Fig. 3). The respective accession numbers were obtained from the BLAST search program of NCBI and the identifiable BspP accession numbers respect to the *B. glumae, B. plantarii, B. gladioli, R. solanacearum* are listed in the following respective order: WP_015878232.1, WP_042629168.1,

WP_124096127.1, WP_080894246.1.

III. Survival of the *bspP* mutant in static and shaking culture conditions

To determine whether the gene sacrifice that caused the mutation in *bspP* provides advantageous adaptive tactics for survival in experimental conditions, I determined the viability of the wild-type strain BGR1, the *bspP* null mutant BGP38, and the *bspP* complementation strain BGP38C at 28°C for 1 week in static culture. Densities of all three strains in static culture increased 3–4 days after incubation, and the BGP38 population was maintained at approximately 1×10^9 CFU/ml up to 7 days (Fig. 4A). However, the population of wild-type BGR1 and the *bspP* complementation strain BGP38C gradually decreased after 4 days of incubation (Fig. 4A). In vigorous shaking culture, wild-type BGR1 and the *bspP* complementation strain BGP38C exhibited normal growth throughout the incubation period; however, population of BGP38 decreased steeply 2 days after inoculation (Fig. 4B). This indicated that survival of the *bspP* mutant was dependent upon culture conditions, particularly oxygen availability.

IV. Physiological characteristics of the *bspP* mutants

To understand how mutations in *bspP* confer improved survival in static culture compared to the wild type, the changes in pH of the static culture were monitored every day for 7 days. Under static culture conditions, the environmental

pH of the *bspP* null mutant BGP38 remained stable at a neutral pH for 7 days (Fig. 5A). However, after 2 days in shaking culture, the environmental pH of BGP38 increased to 8.15 (Fig. 5B). The environmental pH of BGR1 and the *bspP* complementation strain BGP38C showed pH drop to pH 5, from weak alkali pH range within pH 8 then recovered back to neutral pH in both static and shaking culture conditions (Fig. 5B).

To understand how the environmental pH of BGP38 was affected by culture conditions, the oxalate levels produced by BGP38 grown in LB (Fig. 5C) or LB supplemented with HEPES to keep the environmental pH neutral (Fig. 8) with shaking for 2 days were measured. Significantly less oxalate was produced from BGP38 than wild-type BGR1 and the *bspP* complementation strain BGP38C in shaking culture with LB (Fig. 5C). To determine if there was a correlation between the production of oxalate and expression of the oxalate biosynthetic gene *obcA* in supporting the quorum sensing dependent oxalate synthesis for cellular homeostasis by preventing alkali extracellular pH, an estimation in expression levels of *obcA* in wild-type BGR1, the *bspP* null mutant BGP38, and the *bspP* complementation strain BGP38C was made. The *bspP* null mutant BGP38 expressed *obcA* less by 2.5 folds than wild-type BGR1 and the *bspP* complementation strain BGP38C when grown in LB (Fig. 5D). The *bspP* null mutant BGP38 produced both octanoyl- and hexanoyl-L-homoserine lactones to the same levels as those produced by wild-type BGR1 grown in LB supplemented

with HEPES (Fig. 8), which ruled out the possibility that the decreased oxalate production was due to a lack of QS signal production. The phenotypes of the IR-type *bspP* mutant BGS8 were complemented, as shown in Fig. 9.

V. Facilitated pellicle formation and upregulation of *bcsB* expression in the *bspP* null mutant BGP38

In addition to differences in the environmental pH of the *bspP* null mutant BGP38 in static and shaking culture conditions, the key phenotypic difference between wild-type BGR1 and *bspP* null mutant BGP38 was the ability to produce pellicles in static culture (Fig. 6A). The turbidity of cellulase-sensitive pellicles produced by wild-type BGR1, the *bspP* null mutant BGP38, and the *bspP* complementation strain BGP38C after cellulase treatment. Turbidities of cellulase-treated pellicles of BGR1, BGP38, and BGP38C were 0.630 ± 0.067, 1.846 ± 0.062, and 0.812 ± 0.054 (mean ± standard deviation) were measured, respectively (Fig. 6A). These results indicate that the *bspP* null mutant BGP38 produced more cellulose-sensitive pellicles than wild-type BGR1 and the *bspP* complementation strain BGP38C. To confirm that the facilitated pellicle formation in BGP38 was affected by c-di-GMP levels, Imeasured the levels of c-di-GMP levels extracted from wild-type BGR1 and the *bspP* complementation strain BGP38C. Levels of c-di-GMP in BGP38 were significantly higher than those of wild-type BGR1 and the *bspP* complementation strain BGP38C (Fig. 6B). One of

the genes involved in cellulose biosynthesis in *B*. *glumae* was chosen, *bcsB* (BGLU_RS28235), which encodes a putative cellulose synthase regulator, and assessed its expression in the *bspP* null mutant BGP38. Expression of *bcsB* was significantly higher by 2.3 folds in BGP38 compared to the wild-type strain BGR1 and the *bspP* complementation strain BGP38C (Fig. 6C).

VI. The *bspP* mutant was avirulent

To determine whether lifestyle changes in the *bspP* null mutant BGP38 affect fitness and virulence, I inoculated rice plants with BGR1, the *bspP* null mutant BGP38, and the *bspP* complementation strain BGP38C. The ability to colonize rice plants was determined by monitoring CFUs. The *bspP* null mutant BGP38 showed no disease symptoms in the rice sheath whereas wild-type BGR1 and the *bspP* complementation strain BGP38C caused typical symptoms (Fig. 7A). Wild-type BGR1 and the *bspP* complementation strain BGP38C successfully colonized the rice sheath for 9 days whereas the population of the *bspP* null mutant BGP38 decreased significantly after 3 days of incubation (Fig. 7B). These results indicate that the null mutation in *bspP* reduced colonization ability in rice plants, resulting in a failure to induce disease symptoms.

DISCUSSION

Bacteria adapt to an ever-changing environment via genetic, physiological, and behavioral changes, a process known as bacterial environmental adaptation (Boles et al., 2008; West et al., 2016). The genetic and physiological flexibility of bacteria plays an important role in ensuring the most suitable variants are present within a niche, providing an important means of survival in undesirable environments (Brooks et al., 2011; Mena et al., 2008) Experimental conditions such as static culture of aerobic bacteria cause limitations in available resources that pressure bacteria to adapt.

Pellicle formation of *B*. *glumae* BGR1 was the first behavioral change observed in oxygen depriving static culture (Kwak et al., 2020). The present study demonstrated that cells of *B*. *glumae* change genetic elements to transform their physiology in a pellicle-forming microenvironment as a survival strategy. This raises important questions. First, what genetic and/or physiological changes occur at the cellular and environmental levels to increase the survival of aerobic QS bacteria under adverse growth conditions? Second, do these genetic or physiological changes affect QS itself in aerobic QS bacteria? Finally, do these genetic or physiological adaptations for improved survival increase the fitness of pathogenic bacteria in the host?

The results here are considered to have at least partially answered these

questions. Pellicle formation is a collective behavior of *B*. *glumae* that provides a survival advantage in static culture (Kwak et al., 2020). However, the genetic and physiological natures of cells in static culture were not homogeneous. Colony variants in developed pellicles appear to tolerate stressors by forming biofilms as a survival strategy (Déziel et al., 2001; Biswas et al., 2009; Stewart et al., 2008; Li et al., 2016). Similarly, observation of the two types of colony variants, CS- and IR-types, which were considered as a result of adaptation of individual cells in a static bacterial population. The two types of colony variants were consistently observed, and the 94% was the CS-type which prevailed over the IR-type and wild type from the total count of CFU. However, with the exception of colony morphology, the difficulty in distinguishing between the two types in terms of physiological phenotype exists. The IR-type was observed with the deletion mutants BGA1 and BGS8, while the R1S1 mutant, with a deletion of 321 bp near the 3' end of *bspP*, exhibited a CS-type morphology. Because BspP has PAS-9 and SpoIIE domains that are involved in cellular signal processing, colony morphology may vary depending on the location of the mutation. Furthermore, it is unclear whether any signaling process mediated by BspP plays a role in the physiological changes observed in the *bspP* mutant.

Both types of colony variants formed facilitated pellicles compared to wild-type BGR1 and had various mutations in the *bspP* gene in *B*. *glumae*. The results of accelerated pellicle formation and c-di-GMP level measurements were in good

agreement although the role of BspP in the biosynthesis or degradation of c-di-GMP is unknown. In addition to promoting pellicle formation, the environmental pH was sufficiently increased to induce toxicity; this was a result of decreased expression of *obcA* in the *bspP* mutant. It is hard to believe that this reduced expression of *obcA* is a function of QS, because the mutant-generated QS signals were at approximately the same level as the wild type grown in LB supplemented with HEPES. These results suggest that there may be other undiscovered factors associated with BspP that are important for *obcA* expression. Considering that BspP contains domains such as PAS-9 that act as a molecular sensor and play a key role in protein–protein interactions (Henry et al., 2011; Taylor et al., 1999) it is likely that BspP interacts with additional proteins.

Plant pathogenic bacteria that cause leaf disease multiply in apoplasts, which are rich in humidity and air and produce virulence factors (Velásquez et al., 2018; Xin et al., 2016). Thus, the infectious bacterial pathogens recognize the surrounding environment, multiply in the infection court, and finally colonize. Such colonization processes are critical for plant pathogenic bacteria to persist in hosts and produce virulence factors. My results show that a null mutation in *bspP* conferred survival advantages in unfavorable *in vitro* growth conditions and facilitated pellicle formation. We did not expect to see the avirulence from BGP38 inoculated rice plants based on my previous results (Kwak et al., 2020) though we assume that current results suggest BspP may have other roles for the pathogenic

interaction between *B. glumae* and rice plants. However, this change, which provides a survival advantage by sacrificing a specific gene, in this case *bspP*, was not beneficial to *B. glumae in vivo* as a pathogenic bacterium. Because BspP homologs have been identified in other plant pathogenic bacteria such as *B. gladioli*, *B. plantari*, and *R. solanacearum*, it would be interesting to determine whether BspP homologs play similar roles in these bacteria.

Because my results show experimental evolutionary aspects of a typical foliar bacterial pathogen *B. glumae* under artificial static culture, it is worth considering the effects of multiple passes of wild-type BGR1 in rice tissue. In addition, because *B. glumae* has a wide range of hosts, including rice, tomato, pepper, and potato, it will be interesting to investigate how *B. glumae* cells adapt to multiple rounds of inoculation and re-isolation in alternating different hosts (Jeong et al., 2003). How biofilm-producing *B. glumae* adapts to its host is still not known. In this study, we focused more on showing a specific gene (*bspP*) mutation triggering facilitated pellicle formation *in vitro* probably as one of the functions of BspP. In addition to the phenotypic changes we observed *in vitro*, there are possibilities that BspP might have other functions *in vivo* since the *bspP* mutant was less virulent. Therefore, this case is quite different from the phenomenon of persistence occurring in biofilm archetype species *Pseudomonas aeruginosa* during interactions between the pathogen and lung systems in cystic fibrosis patients (Moradai et al., 2007). Such *in vivo* experimental evolution experiments would improve understanding of how

cells of *B*. *glumae* evolve in nature after they encounter diverse host plants.

A conclusion can be made as the unidirectional evolutionary methods, such as specific genetic sacrifice, is to adapt to undesirable *in vitro* growth environments that are not always beneficial for the survival of plant pathogenic bacteria such as *B*. *glumae*.

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Table 1. Strains and plasmids used in this study.

Bacterial strain or plasmid	Characteristics[a]	Source or reference
Strains		
Burkholderia glumae		
BGR1	Wild-type, Rif^R	Jeong et al. (2003)
3-2A	*bspP* spontaneous mutant, point mutation at 3′ end	This study
R1S1	*bspP* spontaneous mutant, deletion mutation at 3′ end	This study
R1S2	*bspP* spontaneous mutant, point mutation at 3′ end	This study
R1S3	*bspP* spontaneous mutant, deletion mutation at 3′ end	This study
R1S4	*bspP* spontaneous mutant, point mutation at 3′ end	This study
R1S10	*bspP* spontaneous mutant, deletion mutation at 3′ end	This study
BGA1	*bspP* spontaneous mutant, deletion mutation at 5′end	This study
BGA2	*bspP* spontaneous mutant, point mutation cluster at 5′ end	This study
BGS8	*bpsP* spontaneous mutant, deletion mutation at 3′ end	This study
BGP38	BGR1, *bspP*::Tn*3-gusA38*	This study

Continued on the following page

Table 2 – *Continued*

BGP38C	BGP38, pPAS1::EZ-Tn5, Tp^R	This study
BGB107	BGR1, *bcsB*::Tn*3-gusA107*	Kwak et al. (2020)
Escherichia coli		
DH5α	F-Φ80d*lacZ*Δ*M15*Δ(*lacZYA-argF*) *U169* endA1 *recA1 hsd1hsdR17*($r_k^- m_k^+$) *deoRthi-1supE44λ*$^-$ *gyrA96 relA1*	Gibco BRL
S17-1	Tra^+, *recA*, Sp^R	Simon et al. (1989)
C2110	*polA*, Nal	Stachel et al. (1985)
Plasmids		
pBluescript II SK(+)	Cloning vehicle; phagemid, pUC derivative, Amp^R	Stratagene
pSShe	Cm^R	Stachel et al. (1985)
pHoKmGus	Promoterless *β*-glucuronidase gene; Km^R Amp^R *tnpA*	Bonas et al. (1989)
pRK2013	Tra^+, ColE1 replicon, Km^R	Figurski et al. (1979)
pLAFR3	Tra^-, Mob^+, RK2 replicon, Tet^R	Staskawicz et al. (1987)
pPAS1	Plasmid harboring bspP with Tp^R	This study

[a] Amp^R, ampcilin resistance; Cm^R, chloramphenicol resistance; Km^R, Kanamycin resistance; Nal^R, nalidixic acid resistance; Rif^R, rifampicin resistance; Sp^R, spectinomycin resistance; Tet^R, tetracycline resistance.

Table 2. Primers used in this study

Oligonucleotide	Sequence (5′ → 3′)[a]
BGLU_RS28885F	GTGAAACGAATCGAAGCGATCATC
BGLU_RS28885R	TCACCGGCCAGCAGGCG
BGLU_RS28235F	CTCGCTGCTGCTGTGCT
BGLU_RS28235R	AGCGTGACCAGCTGGTTGATG
BGLU_RS27005F	AATCGCCGGCATTATTGAA
BGLU_RS27005R	TCGAGCGAATGGTTTCCTCG
BSPP-1F	CGTCGACGCGTTCGTCGC
BSPP -1R	GCAAACGACCCCGGCAGG
BSPP 2F	GAGCTGTACTACTTCTGCACC
BSPP -2R	CAGCAGGTCGAAGCAGCCG
BSPP -3F	GTGCACGACYGCCTGATTGCG
BSPP -3R	GAATTCGAACTACGCGAAGCG
BSPP -4F	CTGACGCCCAACGGCGAC
BSPP -4R	CGCAGGTCCGATGACGAGTC
BSPP -5F	CTGCCGCTGCGCTTCCTGCCGTC
BSPP -5R	GTGTACCGGTTCCAGGCGGCC
BSPP -6F	GARCARGAACACGCCGCATGACAC
BSPP -6R	CACCTTCGTCACCACGCATGC

[a] Restriction sites are not shown.

Figure 1. Spontaneously occurring *B*. *glumae* colony variants were produced in static culture. (A) Population density of colony variants was greater than the population density of wild-type BGR1 at day 3 of incubation in static culture at 28°C. Batch cultures of BGR1 prepared strictly from the static culture from day 0 to 7 at 28°C showed an exponential increase in the population density of colony variants. (B) The evolved colony variants had morphologically different shapes compared to wild-type BGR1: colonies with a crater in the middle (CS-type) and colonies with an irregular boundary (IR-type).

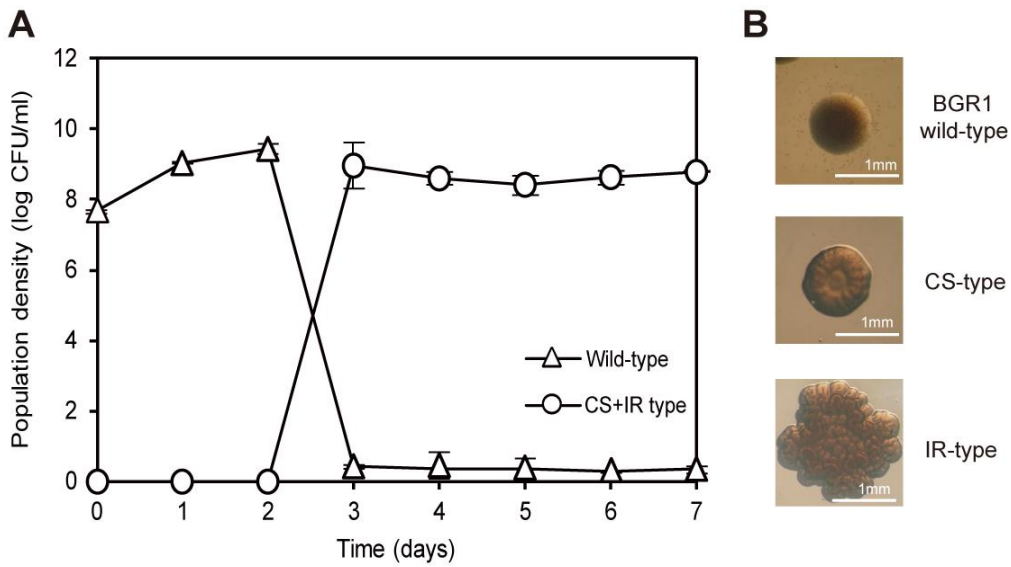

A
B
Population density (log CFU/ml)
Time (days)
Wild-type
CS+IR type
BGR1
wild-type
CS-type
IR-type
1mm

Figure 2. Colony variants in static culture had various mutations in *bspP*. The *bspP* gene consists of 3024 base pairs and putatively encodes 1013 amino acids. Spontaneously evolved IR-type BGA1 and BGS8 had a 95 bp deletion including a putative translation start codon GTG at the 5′ end of *bspP* and a 917 bp deletion at the 3′ end of *bspP*. Additional spontaneously evolved CS-type colonies in static culture had mutations in *bspP* as follows: strains 3–2A, R1S2, and R1S4 had a point mutation at the 3′ end; and strains R1S1, R1S3, and R1S10 had 132, 2, and 1 bp deletions at the 3′ end, respectively. The spontaneously evolved CS-type BGA2 had six point mutations all clustered at the 5′ end of *bspP*. The CS-type BGP38 (*bspP*::Tn*3*-*gusA38*) represents a Tn*3*-*gusA* insertional mutation in *bspP*. A solid black bar represents BspP with the conserved domains PDC2, PAS-9, SpoIIE, and HATPase in small solid black boxes below BspP, a thin arrow below BspP represents the *bspP* gene (3042 bp), | | represents deletion mutation regions, *represents point mutation sites, Δ in parentheses represents the number of nucleotides deleted, → in parentheses represents nucleotide changes, vertical dotted lines with grey shades indicate the positions of domains in BspP, and horizontal waves represent faulty proteins after premature termination. Translation termination is denoted with an arrow (>).

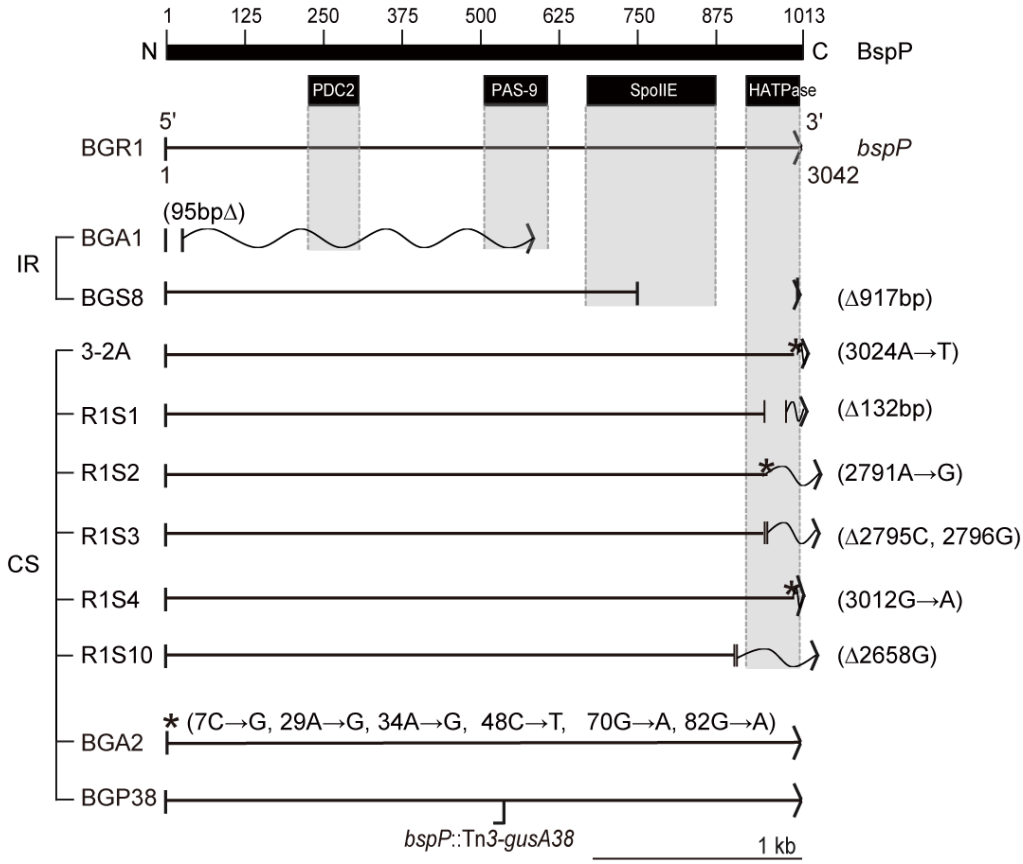

1
125
250
375
500
625
750
875
1013
N
C
BspP
PDC2
PAS-9
SpoIIE
HATPase
5'
3'
BGR1
bspP
1
3042
(95bpΔ)
BGA1
IR
BGS8
(Δ917bp)
3-2A
(3024A→T)
R1S1
(Δ132bp)
R1S2
(2791A→G)
R1S3
(Δ2795C, 2796G)
CS
R1S4
(3012G→A)
R1S10
(Δ2658G)
(7C→G, 29A→G, 34A→G, 48C→T, 70G→A, 82G→A)
BGA2
BGP38
bspP::Tn3-gusA38
1 kb

Figure 3. Conserved domains of BspP in *B*. *glumae* and other plant pathogenic bacteria. The following homologous domains were identified in *B*. *glumae*, *B*. *plantarii*, *B*. *gladioli*, and *R*. *solanacearum* with a heme binding pocket: PDC2, SpoIIE, and HATPase. A sensory box was present in *B*. *gladioli* and *R*. *solanacearum*, and no PAS-9 domain was present in *R*. *solanacearum*. The e-values and accession numbers below the representing domains were obtained from GenBank. The respective accession numbers of BspP for *B. glumae, B. plantarii, B. gladioli,* and *R. solanacearum* are as follows: WP_015878232.1, WP_042629168.1, WP_124096127.1, and WP_080894246.1. The identifiable domain accession numbers of homologous BspP in the four species of interest are as follows: PDC2 (cd12915), PAS-9 (pfam13426), SpoIIE (pfam07228), HATPase (cd16936), sensory-box (TIGR00229).

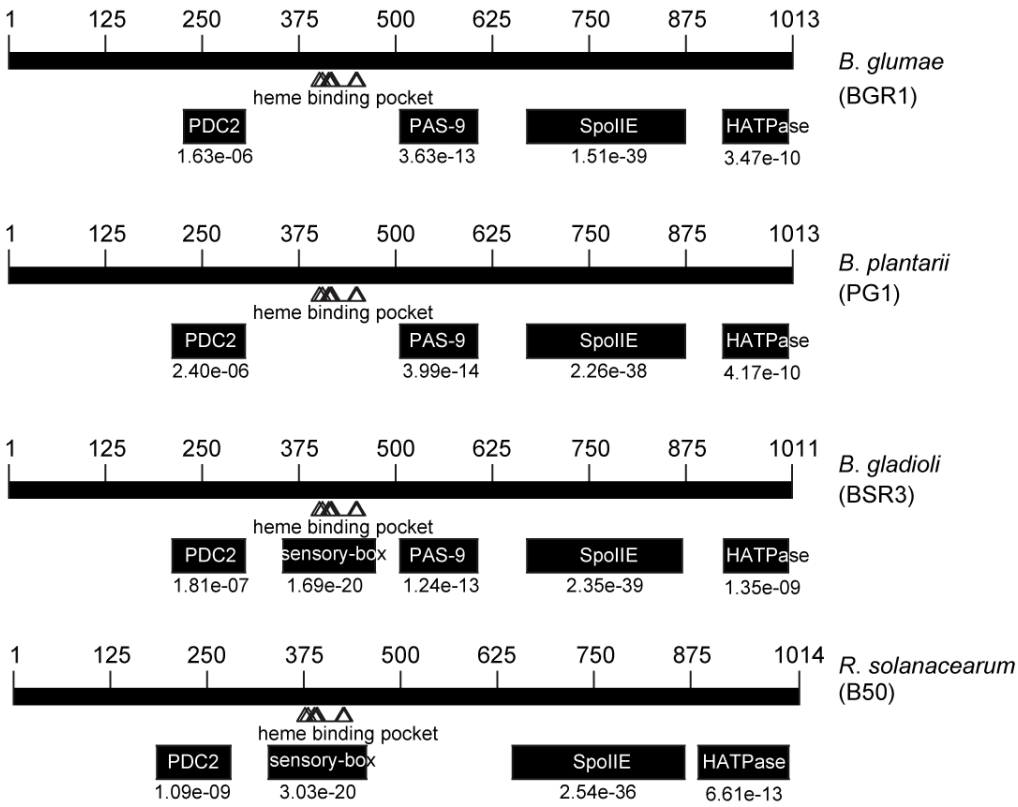

1
125
250
375
500
625
750
875
1013
heme binding pocket
PDC2
1.63e-06
PAS-9
3.63e-13
SpoIIE
1.51e-39
HATPase
3.47e-10
B. glumae
(BGR1)
1
125
250
375
500
625
750
875
1013
heme binding pocket
PDC2
2.40e-06
PAS-9
3.99e-14
SpoIIE
2.26e-38
HATPase
4.17e-10
B. plantarii
(PG1)
1
125
250
375
500
625
750
875
1011
heme binding pocket
PDC2
1.81e-07
sensory-box
1.69e-20
PAS-9
1.24e-13
SpoIIE
2.35e-39
HATPase
1.35e-09
B. gladioli
(BSR3)
1
125
250
375
500
625
750
875
1014
heme binding pocket
PDC2
1.09e-09
sensory-box
3.03e-20
SpoIIE
2.54e-36
HATPase
6.61e-13
R. solanacearum
(B50)

Figure 4. Survival rates of the *bspP* null mutant BGP38 in static and shaking culture. (A) The population density of wild-type BGR1 and the *bspP* complementation strain BGP38C subsided at day 5 after subculture while the *bspP* null mutant BGP38 (*bspP*::Tn*3-gusA38*) gradually increased until day 7 of incubation at 28°C. (B) Population density of the *bspP* null mutant BGP38 dropped steeply at day 3 of incubation whereas wild-type BGR1 and the *bspP* complementation strain BGP38C exhibited normal growth without fluctuation.

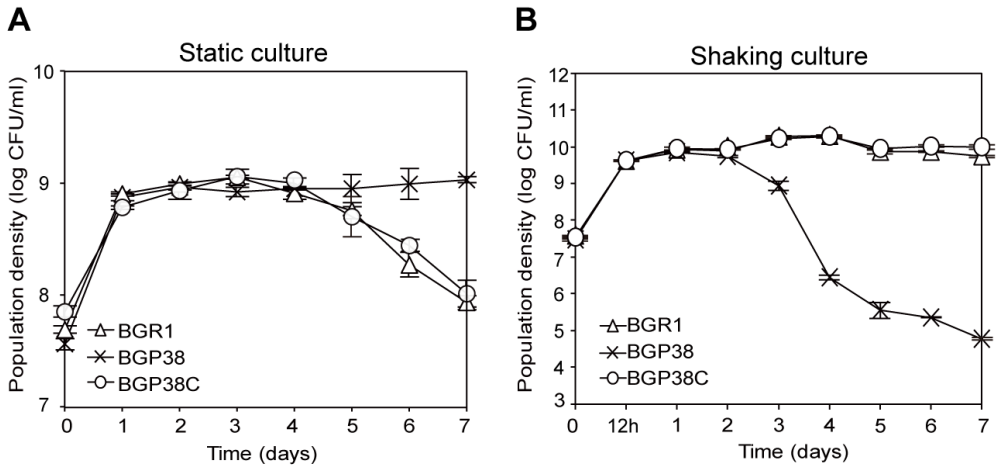

A
Static culture
Population density (log CFU/ml)
Time (days)
BGR1
BGP38
BGP38C
B
Shaking culture
Population density (log CFU/ml)
Time (days)
12h
BGR1
BGP38
BGP38C

Figure 5. Measurements of environmental pH, oxalate levels, and *obcA* expression in the *bspP* null mutant BGP38. (A) The environmental pH of wild-type BGR1 and *bspP* null mutant BGP38 dropped close to 6 at day 5 while the environmental pH of *bspP* null mutant BGP38 was stable at a neutral level from day 0 to 7 in static culture at 28°C. (B) The environmental pH of BGR1 and BGP38C showed normal fluctuation patterns as previously shown in shaking culture whereas the environmental pH of the *bspP* null mutant BGP38 suffered was alkaline. (C) The *bspP* null mutant BGP38 produced less oxalate compared to the levels produced in wild-type BGR1 and the *bspP* complementation strain BGP38C. (D) Expression of *obcA* in the *bspP* null mutant BGP38 was significantly lower than that in wild-type BGR1. The asterisk (*) represents a significant difference ($P < 0.05$) in the normalized fold expression of *obcA* among wild-type strain BGR1, BGP38, and BGP38C.

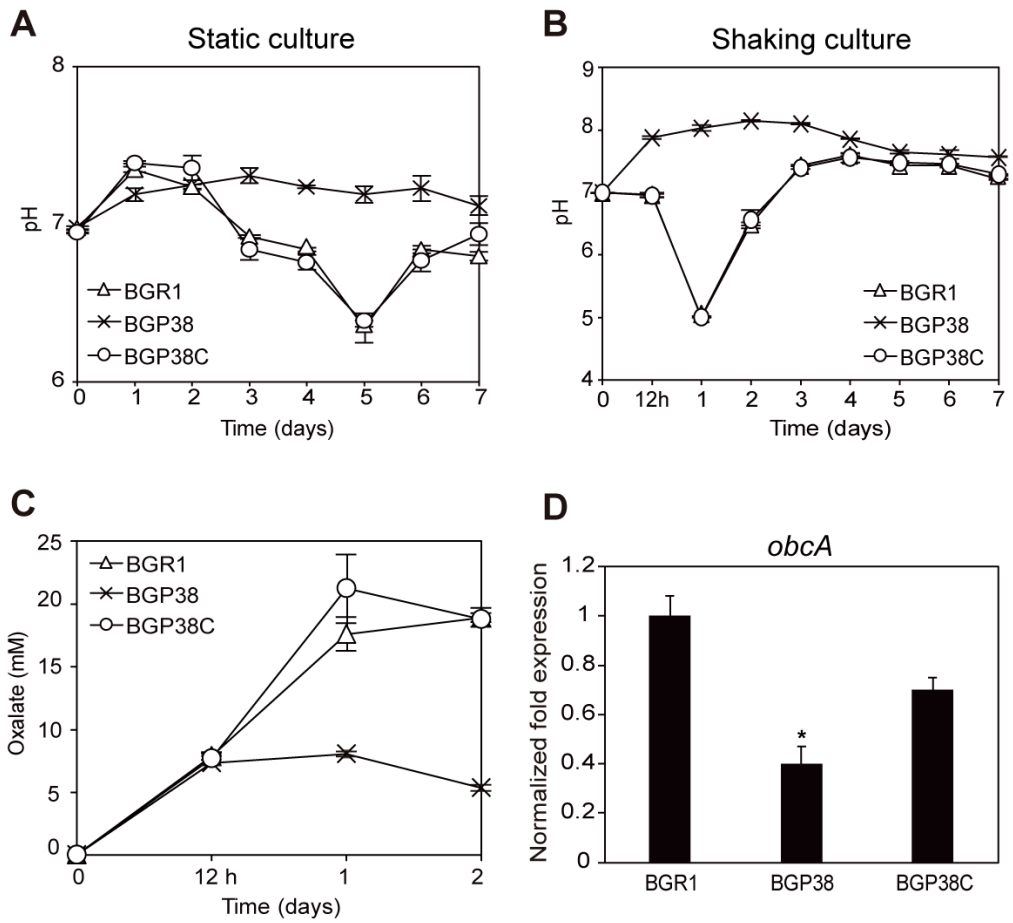

A
Static culture
pH
Time (days)
BGR1
BGP38
BGP38C
B
Shaking culture
pH
Time (days)
BGR1
BGP38
BGP38C
C
Oxalate (mM)
Time (days)
BGR1
BGP38
BGP38C
D
obcA
Normalized fold expression
BGR1
BGP38
BGP38C

Figure 6. Increased level of pellicle and c-di-GMP biosynthesis and upregulated *bcsB* in *bspP* null mutant BGP38. (A) The levels of cellulase-sensitive pellicles were estimated by turbidity following treatment with 0.1% (v/v) cellulase after 3 days of incubation (mean ± standard deviation). (B) The levels of c-di-GMP were elevated in the *bspP* null mutant BGP38 compared to those of wild-type BGR1 and the complementation strain BGP38C. (C) Expression of the bacterial cellulose synthase regulator gene (*bcsB*, BGLU_RS28235) was significantly higher in the *bspP* null mutant BGP38 than in wild-type BGR1 and the *bspP* complementation strain BGP38C. The asterisks (*) represent significant differences (P <0.05) in turbidity after cellulase treatment, c-di-GMP (pmol/10^8 cells), and normalized fold expression of *bcsB* among wild-type strain BGR1, BGP38, and BGP38C.

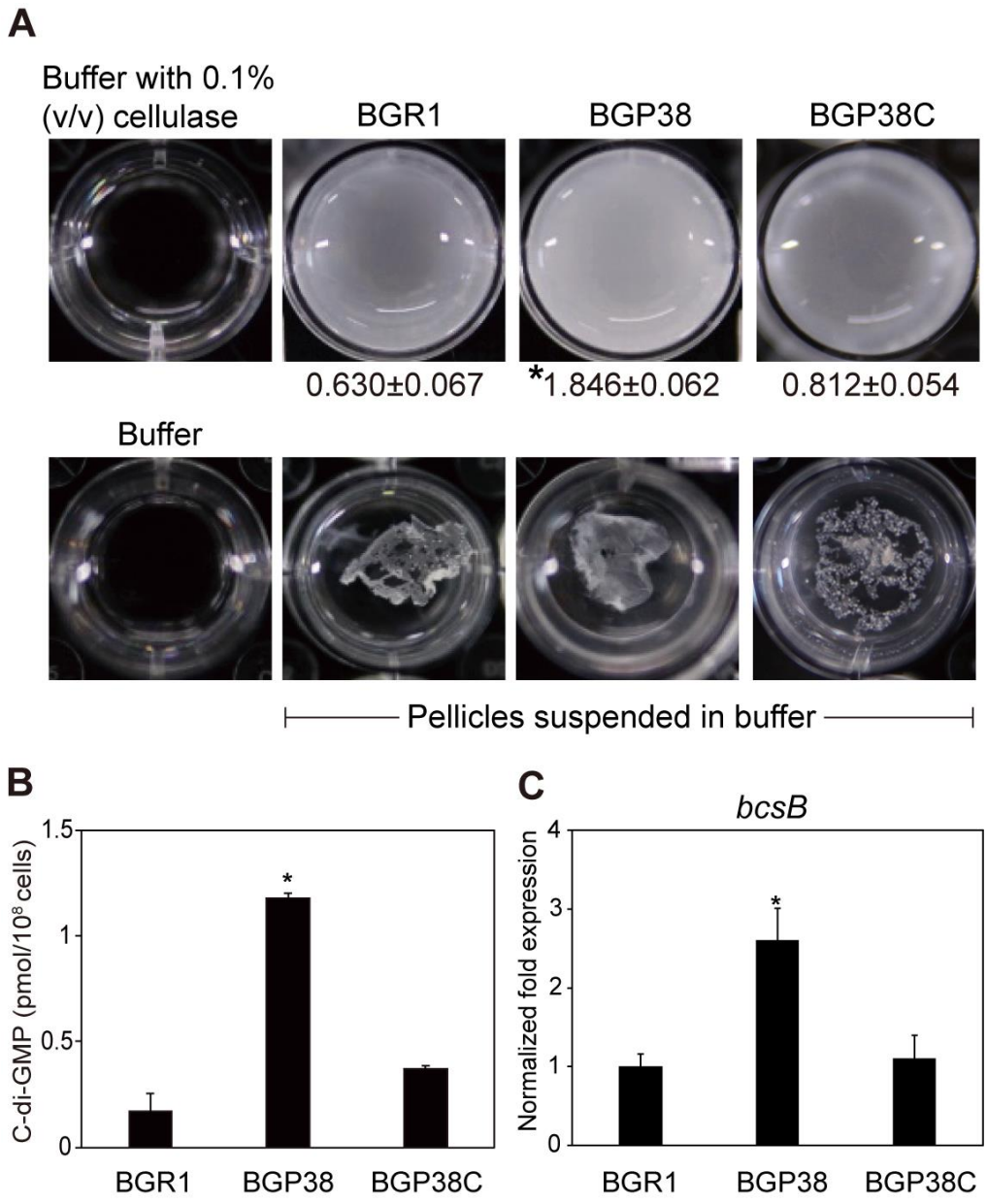

A
Buffer with 0.1%
(v/v) cellulase
BGR1
BGP38
BGP38C
0.630±0.067
*1.846±0.062
0.812±0.054
Buffer
Pellicles suspended in buffer
B
C-di-GMP (pmol/10^8 cells)
1.5
1
0.5
0
*
BGR1
BGP38
BGP38C
C
bcsB
Normalized fold expression
4
3
2
1
0
*
BGR1
BGP38
BGP38C

Figure 7. The *bpsP* mutant BGP38 was avirulent and exhibited less effective colonization than wild-type BGR1. (A) The *bspP* null mutant BGP38 exhibited no visible symptoms whereas wild-type BGR1 and the complementation strain BGP38C caused severe symptoms in rice sheath. (B) The *bspP* null mutant BGP38 failed to colonize effectively in rice sheath after 9 days.

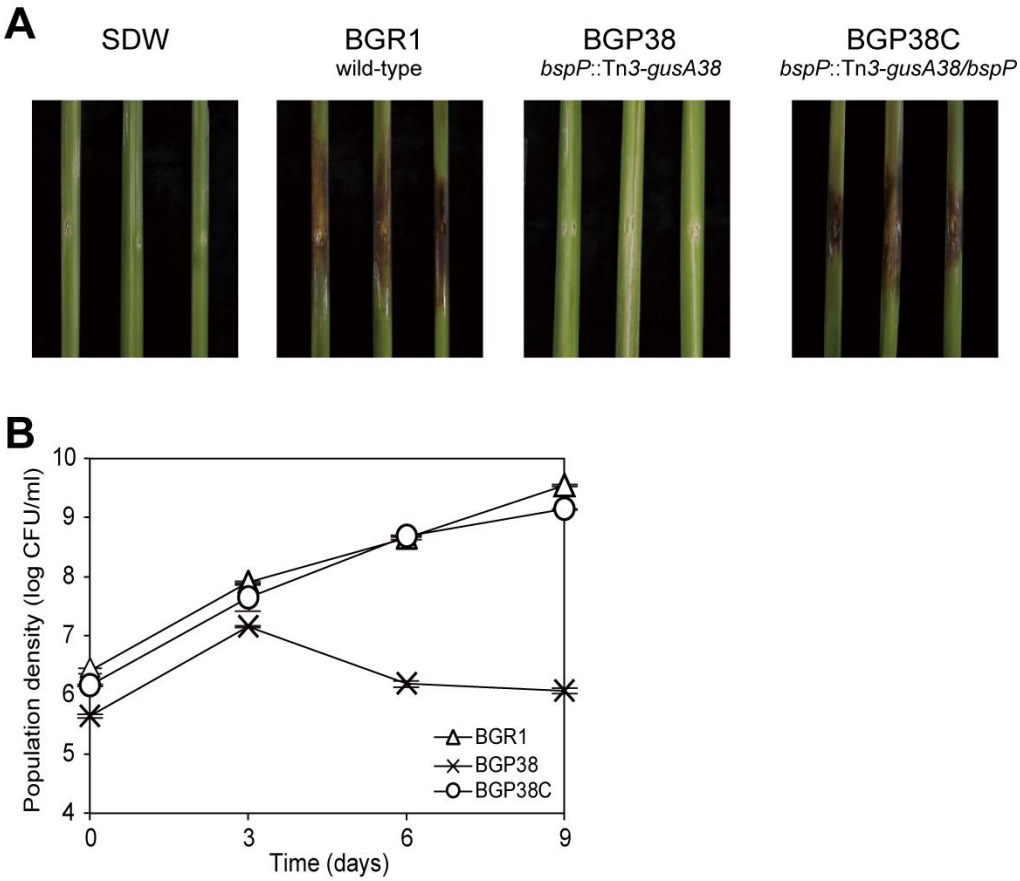

A
SDW
BGR1
wild-type
BGP38
bspP::Tn3-gusA38
BGP38C
bspP::Tn3-gusA38/bspP
B
Population density (log CFU/ml)
10
9
8
7
6
5
4
0
3
6
9
Time (days)
BGR1
BGP38
BGP38C

Figure 8. Autoinducer and oxalate biosynthesis, expression level of *obcA* in the *bspP* null mutant BGP38 during shaking culture of LB supplemented with 100 mM HEPES. (A) Autoinducer assay of wild-type BGR1 and *bspP* null mutant BGP38 revealed no difference between the two strains. (B) Oxalate production in the *bspP* null mutant BGP38 was elevated to the level of wild-type BGR1 and the *bspP* complementation strain BGP38C when cultured in LB supplemented with 100 mM HEPES. (C) Expression of *obcA* in the *bspP* null mutant BGP38 was elevated compared to *obcA* expression in LB culture. The asterisk (*) represents a significant difference ($P<0.05$) in normalized fold expression of *obcA* among BGR1, BGP38, and BGP38C.

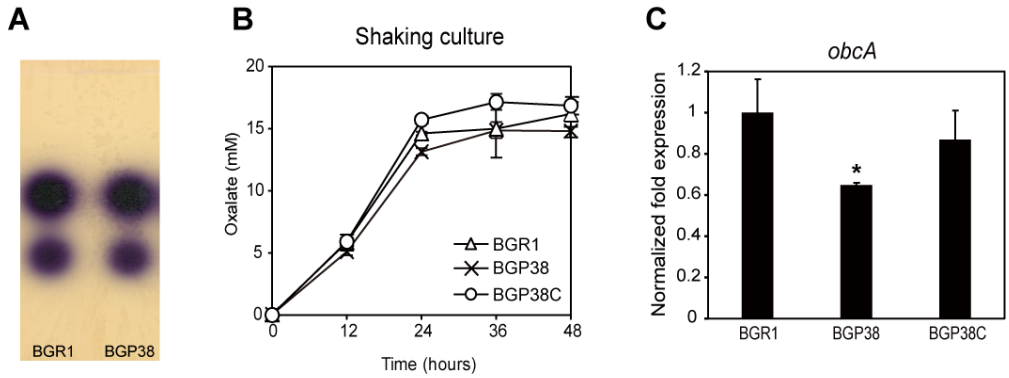
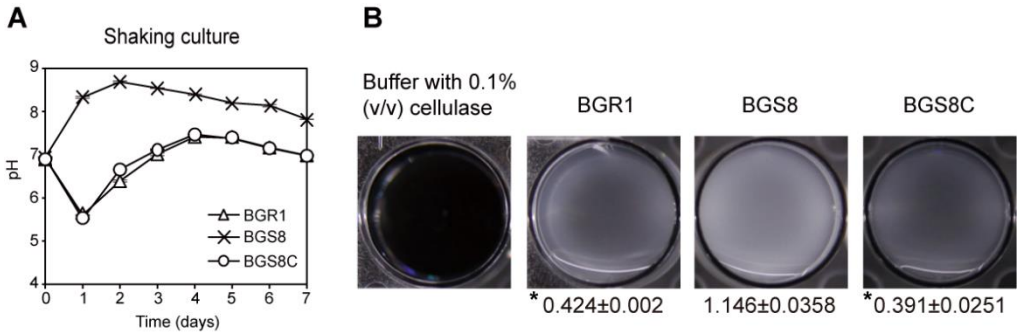

A
BGR1
BGP38
B
Shaking culture
Oxalate (mM)
20
15
10
5
0
0
12
24
36
48
Time (hours)
BGR1
BGP38
BGP38C
C
obcA
Normalized fold expression
1.2
1
0.8
0.6
0.4
0.2
0
*
BGR1
BGP38
BGP38C

Figure 9. Phenotypic complementation of IR-type *bspP* mutant BGS8 with pPAS1. (A) The pH measurement of BGS8C and BGR1 indicate the IR-type colony variant *bspP* mutant BGS8 recovered pH adjusting ability when BGS8 was complemented with pPAS1 that BGS8C pH became neutral while BGS8 pH stayed within the range of weak alkali pH 8. (B) The turbidity of cellulase treated harvested pellicles of BGR1, BGS8, and BGS8C indicate the dense pellicle formation in *bspP* mutant IR-type BGS8 and when complemented with pPAS1, reduced cellulose production to wild-type BGR1. The respective accession numbers of the domains were obtained from the NCBI BLAST search program. The asterisk (*) represents a significant difference ($P<0.05$) in turbidity between wild type BGR1, BGS8, and BGS8C.


A
Shaking culture
pH
Time (days)
BGR1
BGS8
BGS8C
B
Buffer with 0.1%
(v/v) cellulase
BGR1
BGS8
BGS8C
*0.424±0.002
1.146±0.0358
*0.391±0.0251

세균벼알마름병원균 *Burkholderia glumae*의 정치배양시 발생하는 적응 돌연변이와 펠리클 형성

곽지영

초록

세균벼알마름병원균은 호기성 식물 병원균으로써 호의적이지 않은 환경에서 생존하기 위해 운동성 세균 생활방식을 고착성 세균 생활방식으로 전환하고 유전자 희생을 통한 세균막을 형성 후 생존한다. 유전 및 표현형의 환경 적응을 위한 변이는 환경에 가장 적합하게 변이 되는데, 호기성인 세균벼알마름병원균 야생형 BGR1은 공기 순환 없는 액체 배양 조건에서 편모 의존적 운동성으로 대기와 가까운 액체의 경계 면으로 이동하여 생존하고 빠르게 세균막을 이룬다. 대기와 배양액의 경계 면에 도달한 BGR1은 운동성을 잃고 세균막을 이루는 세균 군집의 모양은 고체 배지 배양 시 각기 다르게 나타난다. 촉진된 세균막은 bis-(3′-5′)-cyclic dimeric guanosine monophosphate(c-di-GMP)의 생합성이 증가하여 이루어진 현상으로 호의적이지 않은 생체외 시험관적 조건에서 생존하기 위한 적응이다. 생체외 시험관 적 조건에서 생존하기위한 적응이 성공적으로 BGR1이 살 수 있도록 도움이 되었지만 생체내, 벼에 접종시 병원성이 현저히 감소되거나 사라진 것이 확인되었다.

주요어: 세균벼알마름병, 펠리클, 환경적응, 셀룰로오스 생합성, 군집형성, 병원성