농학박사 학위논문

세균성벼알마름병원균의 GluS-GluR Two-Component System 의 기능 연구

Multifunctional Roles of GluS-GluR Two-Component Regulatory System in *Burkholderia glumae*

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마룬가 조안

Multifunctional Roles of GluS-GluR Two-Component Regulatory System in *Burkholderia glumae*

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Marunga Joan

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Multifunctional roles of GluS-GluR two-component regulatory system in *Burkholderia glumae*

UNDER THE DIRECTION OF DR. INGYU HWANG

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

BY
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
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Multifunctional roles of GluS-GluR two-component regulatory system in *Burkholderia glumae*

Marunga Joan

ABSTRACT

Burkholderia glumae, just like any other microorganism, has a variety of adaptable biological systems that provide insight into how these organisms evolve, adapt, and function in a variety of environments. Despite the complexity of some of these systems, this work sheds light on the two-component regulatory systems (TCSs) paradigm, which serves as the basis for information flow throughout bacteria. Random mutagenesis of *B. glumae* BGR1 with mini-Tn*5* resulted in a cell filamentation in Luria–Bertani (LB) medium in one of the mini-Tn*5* derivatives. Molecular and genetic analysis revealed that *gluR* (BGLU 1G13360), a two-component system response regulator gene, carried the mini-Tn*5* insertional mutation. A putative sensor kinase, *gluS* (BGLU 1G13350), was found downstream of *gluR*, prompting an exploratory study of the GluS-GluR TCS functional roles in *B. glumae* BGR1. The *gluR* mutant, unlike the *gluS* mutant formed filamentous cells in LB medium, was sensitive to 42°C, and the expression of genes responsible

for cell division and cell-wall (*dcw*) biosynthesis were elevated at transcription levels compared to the wild type, classifying GluR as an essential regulatory factor for cell division. TCSs regulate a variety of bacterial activities via an organized system in which the sensor kinase passes environmental cues to the response regulator, which decodes an appropriate cellular response. Accordingly, this study identified glutamine and glutamate as extrinsic cues that initiate cell division in *B. glumae* via GluR. Notably, GluR, and not GluS was also required for elicitation of the hypersensitive response in tobacco leaves, full virulence in host rice plants, and detoxification of hydrogen peroxide; all of which are important factors in the pathogenicity, survival, and fitness of *B. glumae*. GluR directly interacts with the type III secretion system and a manganese catalase gene *katM* to promote virulence and fitness of the pathogen. This study further showed that GluS-GluR is a functional TCS pair regulating β – lactam antibiotic resistance of *B. glumae*, but through a distinct mechanism. The inactivation of *gluS* or *gluR* conferred resistance against β-lactam antibiotics, whereas the wild type was susceptible to those antibiotics. This phenotype was supported by the significantly increased expression of genes encoding metallo-β-lactamases and penicillin-binding proteins in the TCS mutants compared to those in the wild type. Overall, this study adds to our understanding of how TCSs affect

bacteria's sophisticated molecular systems, gives a new perspective on antibiotic resistance processes, and may provide a novel therapeutic approach for the successful control of bacterial pathogens.

Keywords: *Burkholderia glumae*, two-component system, GluR, GluS, cell division, virulence, β –lactam resistance, type III secretion system

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INTRODUCTION

Burkholderia glumae is a Gram-negative, non-fluorescent, rod-shaped bacterium with a polar flagellum (Cho *et al.*, 2007). This bacterium was discovered in the early 1950s (Goto and Ohata, 1956), came to prominence as a rice bacterial pathogen in 1967 (Kurita, 1967), and was later classified as the major cause of rice bacterial panicle blight (PBP). It is characterized by the ability to infect various stages of crop development (Pedraza *et al.*, 2018) instigating about 70% yield loss in heavily infested fields (Zhou-qi *et al.*, 2016). *B. glumae* relishes in the rice optimal growing conditions of warm nights and high humidity, resulting in spikelet, floret, and seeds sterility, grain discolorations, and grain weight loss (Jeong *et al.*, 2003; Xie *et al.*, 2003). Since inception, *B. glumae* has emerged as one of the most serious threats to the global rice production, particularly in tropical and sub-tropical countries (Ham *et al.*, 2011). To date, this rice pathogen has been reported in over 25 countries (Compendium, 2020) of rice-growing regions of Africa, Asia, South and Central America (Ham *et al.*, 2011; Zhou-qi *et al.*, 2016).

Not only is *B. glumae* a problem of rice, but it has also been linked to bacterial wilt in tomato, pepper, eggplant, and sesame plants (Jeong *et al.*, 2003). In addition, Jeong *et al.* (2003) discovered that *B. glumae* isolates from the field crops can infect rice plants. Furthermore, strains of this precarious pathogen can also infect humans causing chronic granulomatous disease (Weinberg *et al.*, 2007), as well as act as

opportunistic pathogens in cystic fibrosis patients (Coenye and Vandamme, 2003). Interestingly, when inoculated into rice plants, *B. glumae* AU6208 strain obtained from granulomatous patients was found to be more virulent than other *B. glumae* strains (Devescovi *et al*., 2007), presenting a varied array of inoculum sources.

Due to the pliability of *B. glumae,* some rice growing countries like China and Indonesia have classified this bacterium as a quarantine pathogen to restrict its entry into their agroecosystems during international trade (Zhou-qi *et al.*, 2016; Safni and Lubis, 2019). With the growing economic importance of *B. glumae*, the quest to address this pathogen is still on-going. The severity of BPB is facilitated by the diverse pathogenic strains and lack of effective methods to control the disease or pathogen. One of the most sought-after method is raising resistant varieties, however, only partially resistant varieties are currently available and are said to lack the desired commercial characteristics (Sayler *et al.*, 2006; Ham and Groth, 2011). Attempts to develop efficient control measures have spurred numerous studies, particularly on the processes underlying pathogenicity, with the hope that a deeper understanding of molecular mechanisms may lead to the development of better disease or pathogen control strategies.

The pathogenesis of *B. glumae* is a complex process involving multiple virulent factors (Zhou-qi *et al.,* 2016). Among these, phytotoxins (Kim *et al.*, 2004) and lipases (Frenken *et al.*, 1993) are classified as the major virulent factors (Zhou-qi *et al.*, 2016). Studies have linked the control of these virulent factors to biological

systems such as the quorum sensing (QS), type III secretion systems, and two-component systems (TCSs). The QS system, in particular, has attracted a significant amount of attention that unveiled its involvement in most virulence factors of the pathogen. A good example has been demonstrated in the production and expression of phytotoxins (Schuster and Greenberg, 2006). *B. glumae* produces bright yellow phytotoxins known as toxoflavins and fervenulin (Kim *et al.*, 2004). And while the biosynthesis of fervenulin remains unclear, that of toxoflavins has been well characterized and found to be under the control of the QS system (Kim *et al.*, 2004). Briefly, toxoflavin biosynthesis and transportation is controlled by Tox operons comprised of five genes dubbed *tox A, B, C, D,* and *E* and four genes *tox F,* G, H, and I, respectively. It has also been established that the expression of both *toxABCDE* and *toxFGHI* operons is regulated by a LysR-type regulator – ToxR (Kim *et al.*, 2004; Suzuki *et al.*, 2004). Furthermore, Kim *et al.* (2004) showed that the expression of the *toxABCDE* and *toxFGHI* operons requires a transcriptional activator ToxJ whose expression is regulated by a QS system comprised of TofI, a LuxI family protein and its cognate receptor TofR, a LuxR family protein. Other virulent factors that are said to contribute to the full virulence of *B. glumae* include, but not limited to, the flagellar motility system, type III secretion systems (Kang *et al.*, 2008), exoploysaccharides, and endopolygalacturonas (Jeong *et al.*, 2003), which are also said to be intricately linked with the QS system (Zhou-qi *et al.*, 2016). It is important to note that *B. glumae* contains only one QS system, TofI/TofR (Kim

et al., 2004). This poses a question of sufficiency and thoughts of other systems that are yet to come into the limelight.

Two-component systems (TCSs) are important mediators of bacterial signal transduction. Bacteria are continually bombarded with different molecules from their environment or metabolic biproducts, and they are able to mount an appropriate response via TCSs (Hoch, 2000). The essence of TCS signal transduction involves the transfer of signal recognition to gene activation or other cellular responses, ultimately evoking an adaptive response. TCSs are typically composed of a membrane-located sensor kinase and a cytoplasmic transcription response regulator (Gao and Stock, 2009). Generally, the sensor kinases are autophosphorylated after sensing an environmental stimulus, followed by phosphotransfer from the phosphorylated sensor kinases to the response regulators (Gao and Stock, 2009). The phosphorylated response regulators then undergo conformational changes to become active, thereby controlling the expression of target genes (Gao and Stock, 2009).

Although compelling evidence has shown that two-component systems (TCSs) ably coordinate and regulate processes like virulence, drug resistance, growth, and viability (Throup *et al.*, 2000; Tiwari *et al.*, 2017), there have been limited studies on their involvement in *B. glumae*. To date, one TCS system - PidR/PidS has been reported to be essential in pigmentation and virulence properties of *B. glumae* 411 gr-6 strain (Karki *et al.*, 2012). Because studies have shown that *B. glumae* strains

exhibit uniqueness following the regions and habitats, in this study, I set out to investigate the role of two-component regulatory systems in *B. glumae* BGR1 strain isolated from rice samples in South Korea.

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CHAPTER 1

The GluR Response Regulator is Required for Cell Division in the Rice Pathogen *Burkholderia glumae*

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ABSTRACT

Bacterial two-component regulatory systems (TCS) coordinate physiological functions by controlling the expression of genes in response to environmental cues. *Burkholderia glumae*, like most bacteria pathogens, has a plethora of TCSs. To gain a better understanding of this infamous pathogen, I assessed the functionally significant TCS(s) present in this pathogen. Through molecular and genetic analysis, I discovered that the insertion of mini-Tn*5* in *gluR* resulted in cell filamentation in Luria–Bertani medium. In this chapter, I show that a genetically linked but functionally unpaired two-component system composed of the sensor kinase GluS (BGLU_1G13350) and the response regulator GluR (BGLU_1G13360) is required for cell division in the rice pathogen *B. glumae* BGR1. The *gluR* null mutant, unlike the *gluS* mutant, formed filamentous cells in LB medium and was sensitive to exposure to 42°C. Expression of genes responsible for cell division and cell-wall (*dcw)* biosynthesis in the *gluR* mutant was elevated at transcription levels compared to the wild type, resulting in an imbalance between FtsZ and FtsA. GluR-His bound to the putative promoter regions of *ftsA* and *ftsZ*, indicating that repression of the septation genes in the *dcw* cluster by GluR is critical for cell division in *B. glumae*. The *gluR* mutant did not form filamentous cells in M9 minimal medium, whereas exogenous addition of glutamine or glutamate to the medium induced filamentous cell formation. These results proposed that glutamine and glutamate may serve as

external stimuli in GluR-mediated cell division in *B. glumae*, suggesting that GluR controls cell division of *B. glumae* in a nutrition-dependent manner. These findings provide insight into how the recognition of external signals by TCS affects the sophisticated molecular mechanisms involved in controlling bacterial cell division.

INTRODUCTION

Two-component systems (TCS) consisting of a sensor kinase and a cognate response regulator are common in bacteria (Gao and Stock, 2009). They are essential for the responses of bacteria to changes in environmental factors such as pH, osmotic pressure, antibiotics, and quorum-sensing (QS) signals (Gao and Stock, 2009). The sensor kinases are autophosphorylated after sensing an environmental stimulus, followed by phosphotransfer from the phosphorylated sensor kinases to the response regulators (Gao and Stock, 2009). The phosphorylated response regulators then undergo conformational changes to become active, thereby controlling the expression of target genes (Gao and Stock, 2009). The genes encoding sensor kinases and response regulators are often genetically linked in bacterial genomes and functionally paired (Stenson *et al*., 2005; Coutte *et al*., 2016). In addition to paired TCSs, sensor kinases and transcriptional regulators can crosstalk, thus modulating multiple biological processes in response to environmental signals irrespective of their genetic linkage (Hellingwerf, 2005; Yamamoto *et al*., 2005; Coutte *et al*., 2016; Chen *et al*., 2017).

Over the years, our group has discovered different factors relating to the social behavior and host interactions of the rice bacterial pathogen *Burkholderia glumae*, which causes rice panicle blight (Kim *et al*., 2004; Goo *et al*., 2015). A phytotoxin, toxoflavin, is the major virulence factor of *B. glumae* and exerts a toxic effect on

photosynthetic organisms by generating radicals under light (Kim *et al*., 2004; Koh *et al*., 2011). The virulence-factor biosynthesis and motility of *B. glumae* are dependent on QS (Kim *et al*., 2004; Kim *et al*., 2007).

In addition to QS, we are interested in TCSs in *B. glumae* BGR1 because they coordinate and regulate the expression of genes critical for adaptation to stress, survival, fitness in the host, and virulence (Uhl and Miller, 1996; Perraud *et al*., 1998; Groisman, 2001; Bronner *et al*., 2004; Loui *et al*., 2009; Freeman *et al*., 2013; Yan *et al*., 2020). For instance, CpxAR of *Actinobacillus pleuropneumoniae* (Yan *et al*., 2020), ArcAB of *Escherichia coli* (Loui *et al*., 2009), and KdpDE (Freeman *et al*., 2013) and PhoPQ (Groisman, 2001) in a variety of bacterial taxa reportedly promote growth, fitness, and survival in the host. In addition, AgrAC, SsrAB, SaeRS, and ArlRS of *Staphylococcus aureus* and BvgAS of *Bordetella pertussis* are necessary for virulence (Uhl and Miller, 1996; Perraud *et al*., 1998; Bronner *et al*., 2004). Few studies have focused on TCSs in *B. glumae*, probably because of concern over repeating works on other pathogens. However, Karki *et al.* (2012) reported that the PidS/PidR TCS is essential for the pigmentation and virulence of *B. glumae* 411gr-6.

In this chapter, I identified a TCS composed of the sensor kinase GluS and the response regulator GluR, which was critical for normal cell division in *B. glumae* BGR1. *gluR* and *gluS* were co-transcribed, but GluR functioned independently of GluS in normal cell division. I show that GluR is important for the regulation of the

gene cluster involved in cell division and cell wall (*dcw*) biosynthesis to maintain balanced expression of FtsZ and FtsA for normal septum formation. Based on the fact that glutamine or glutamate affects cell division control by GluR, I conclude that external nutritional conditions affect cell division control in a TCS-dependent manner in *B. glumae*. These findings have revealed an important understanding of how the recognition of external signals by TCS affects sophisticated molecular mechanisms involved in controlling bacterial cell division.

MATERIALS AND METHODS

I. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, the strains were grown in Luria–Bertani (LB) medium containing 0.1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, and 1.5% agar as required (Affymetrix, Cleveland, OH) with the appropriate antibiotics at 37°C. Antibiotics were used at the following concentrations: rifampicin, 100 μg/ml; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml; kanamycin, 25 and 50 μg/ml; and spectinomycin, 50 μg/ml. 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) was added at 40 μg/ml as necessary.

II. DNA manipulation and sequencing

Basic DNA manipulation was conducted following standard protocols (Sambrook *et al.*, 1989). Plasmid DNA from *E. coli* was isolated using the Biomedic Plasmid DNA Miniprep Kit (Ibiomedic, Korea) following the manufacturer's instructions. DNA sequencing was performed by Macrogen, Inc. (Seoul, Korea). The genetic information and gene IDs for DNA construction were obtained from the *B. glumae* BGR1 genome database (GenBank accession numbers:

CP001503–CP001508; kropbase.snu.as.kr/cgi_bg.cg). I used a previously constructed cosmid library of *B. glumae* BGR1 (Kim *et al*., 2004).

III. Rescue *mini*-Tn*5*, Tn*3-gusA*, and marker-exchange mutagenesis

Using *E. coli* S17-1 (pRescue *mini*-Tn*5*), random mutations were created in *B. glumae* BGR1 as described previously (De Lorenzo *et al*., 1990). Successful mutants were isolated by selection on LB agar containing kanamycin. The rescued *mini*-Tn*5* mutants were screened for phenotypic changes under the microscope. Following a previous method (Kwon and Ricke, 2000), the flanking regions were sequenced using the O-end primer (5′-GGTTTTCACCGTCATCACCG-3′), and the TCS genes were disrupted using the identified rescue *mini*-Tn*5* insertions. The selected mini-Tn*5rescue* mutant, RT271, was complemented by tri-parental mating (Figurski and Helinski, 1979) using pBGH1 plasmid to generate RT271C.

The pLAFR3 derivatives of pBGH1 carrying *gluR* (BGLU_1G13360) and *gluS* (BGLU_1G13350) were mutagenized using Tn*3-gusA* as previously described (Bonas *et al*., 1989). The Tn*3-gusA* insertion site and orientation in each mutant were mapped by restriction enzyme digestion analysis, and the plasmid sequenced using the Tn*3*gus primer (5′-CCGGTCATCTGAGACCATTAAAAGA-3′). The plasmids carrying Tn*3-gusA* insertions were marker-exchanged into *B. glumae* BGR1 via tri-parental mating (Figurski and Helinski, 1979) to generate BGLUR133

and BGLUS35. All marker-exchange mutants were confirmed by southern hybridization analysis.

I used the pBGH13 plasmid, a derivative of pBGH1, to complement the *gluR* mutant. First, pBGH1 DNA was digested with *Sca*I followed by ligation into pBluescript II SK (+). The resulting plasmid DNA was cut with *Bam*HI and *Hin*dIII followed by ligation into pLAFR3, resulting in pBGH13. The pBGH13 was introduced into BGLUR133 via tri-parental mating (Figurski and Helinski, 1979) to produce BGLUR133C.

IV. Bacterial growth and viability assay

Overnight liquid cultures of the *B. glumae* strains were adjusted to an OD_{600} of 0.05 and sub-cultured into fresh LB medium. The cultures were incubated for 30 hours at 37°C with shaking at 250 rpm. At 6-hour intervals, bacterial growth was assayed by spotting 10 μl of serial dilutions in triplicate on LB agar plates. Bacterial growth was expressed as log CFU/ml after 2 days of incubation at 37°C.

Cell viability was assayed using the LIVE/DEAD BacLight Bacterial Viability Kit, which contains SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Fluorescence images were captured using a confocal laser scanning microscope (Leica SP8X, Wetzlar, Germany) at

excitation/emission wavelengths of 483/490–540 and 535/890–680 nm for green and red fluorescence, respectively.

V. Transmission electron microscopy

Bacterial cells were harvested from overnight cultures and prepared for observation by transmission electron microscopy (TEM), as reported previously (Kang *et al*., 2017). Electron micrographs were acquired using a JEM 1010 microscope (JEOL, Tokyo, Japan) with acceleration voltages of 180 and 100 kV from a LIBRA 120 energy-filtration microscope (Carl Zeiss, Oberkochen, Germany).

VI. Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from *B. glumae* BGR1, BGLUR133, and BGLUR133C using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genomic DNA was removed using DNase I (Thermo Fisher Scientific, Vilnius, Lithuania). From 1 μg of RNA, reverse transcription for cDNA synthesis was performed at 42°C for 1 hour with the Recombinant RNasin and M-MLV Reverse transcriptase following manufacturer's instructions (Promega, Madison, WI). Using specific primer sets (Table 2), *ftsA*, *ftsB*, *ftsI*, *ftsK*, *ftsL*, *ftsQ*,

ftsW, and *ftsZ* cDNAs were synthesized. Transcription levels were determined using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) under the following conditions: 95°C for 30 seconds followed by 30 cycles of 95°C for 5 seconds and 55°C for 5 seconds. Using SensiFast SYBR No-ROX Kit (Bioline, Meridian Bioscience, USA), PCR was performed in triplicate, and gene expression values were normalized to 16S *rRNA* using Bio-Rad CFX Manager software.

VII. Constitutive expression of *ftsA* gene

To express the *ftsA* gene under the control of the *trc* promoter in pKK38, I amplified the *ftsA*-coding region from the *B. glumae* strains: BGR1, BGLUR133, and BGLUR133C using primers FtsA_Nco1(5'-GGCCATGGAGCAAAGACTACAAAGATCT-3') and FtsA_HindIII (5'-CCAAGCTTTCAGAAATTGCTCAGGAACC-3') and Takara PCR kit (Takara Bio Inc., Kusatsu, Shiga, Japan) following the instructions of the manufacturer. The PCR fragments were first cloned into the *Sma*1 sites of pBluescript II SK (+) and transferred to *Nco*1-*Hind*III sites of pKK38 following previously described procedures (Kang *et al*., 2017). Using tri-parental mating, pKK38 derivatives were conjugated into their respective *B. glumae* strains to generate strains with elevated *ftsA* genes (BGR1(pFtsA), BGLUR133(pFtsA), and BGLUR133C(pFtsA).

VIII. Growth and viability of *B. glumae* strains at 42°C

The *B. glumae* BGR1, TCS null mutants, and BGLUR133C strains were cultured overnight at 37°C and the optical density at 600 nm (OD_{600}) was adjusted to 0.05. The strains were incubated at 42°C with shaking at 250 rpm for 24 hours in LB and M9 minimal media (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl in 1 l of deionized water containing 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$, supplemented with 0.2% glucose), and the cell density was measured at 6-hour intervals.

IX. Environmental stimuli driving GluR responses

The wild type, *gluR* mutant BGLUR133, and BGLUR133C were cultured in M9 minimal medium. To evaluate whether amino acids are required for GluR activity, M9 minimal medium was supplemented with 10% Bacto Casamino Acids (Becton, Dickson & Co., Franklin Lakes, NJ) that comprises 20 essential amino acids. Individual amino acids (Sigma Aldrich, St. Louis, MI) were analyzed at the concentrations in LB medium (Sezonov *et al*., 2007).

X. Glutamate utilization in *B. glumae*

Overnight liquid cultures of the wild-type BGR1 were adjusted to an optical density of OD_{600} of 0.05 and sub-cultured in LB medium for 24 hours at 37°C with shaking at 250 rpm. At 3-hour intervals, the cultures were centrifuged (14,000 rpm, 4°C, 10 minutes), and the supernatants were collected. Glutamate analysis was carried out by liquid chromatography-mass spectrometry (LCMS-2000, Shimadzu, Japan) at the National Instrumentation Center and Environment Management (Seoul National University, Seoul, Korea).

XI. Scanning electron microscopy

B. glumae strains cultured overnight in LB or M9 minimal medium with/without amino acids were harvested, fixed with Karnovsky's fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.4)), and post-fixed with 1% sodium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at 4°C as described previously (Morris, 1965). Before imaging, the samples were coated with platinum at 10 mA for 270 seconds using a G20 Ion Sputter Coater (GSEM Co., Suwon, Korea) and electron micrographs were acquired using a Carl Zeiss microscope (Auriga, Zeiss Germany).

XII. Electrophoretic mobility shift assay (EMSA)

The coding region of *gluR* was amplified from BGR1 chromosomal DNA with the primers, gluR_Nde1-F and gluR_BamH1-R (Table 2), and then cloned into the *Nde*I and *Bam*HI sites of pET21b (Invitrogen) to produce pGluR-His. GluR-His was overexpressed in *E. coli* strain BL21 (DE3) followed by purification using a Ni-NTA spin column in a buffer containing 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl as described by the manufacturer (Qiagen). With primer sets ftsAp-F/R and ftsZ-F/R (Table 2), the promoter regions of the putative GluR targets, *ftsAp* and *ftsZp*, respectively, were amplified. The resulting PCR products were labeled with biotin using Lightshift Chemiluminescent Electrophoretic Mobility Shift Assay Kits, as described by the manufacturer (Pierce, Appleton, Wisconsin). I used 329 bp upstream of *katE1* as a nonspecific competitor DNA amplified using KatE1-F and KatE1-R primers (Table 2). Purified GluR-His (0.75 μM) was incubated in binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% (v/v) glycerol) containing 1 nM biotin-labeled DNA as described previously (Kim *et al*., 2007). For competition assays, unlabeled target DNA at 20-fold molar excess was added to each reaction with the labeled DNA. Using 4% (w/v) polyacrylamide gels, the reactions were separated and transferred to nitrocellulose membranes. The bands were detected using streptavidin/horseradish peroxidase-derived

chemiluminescence kits, as described by the manufacturer (Pierce) and visualized using ChemiDoc XRS+ and Image Lab Software (Bio-Rad).

XIII. Statistical analysis

All experiments were conducted in triplicate with the appropriate controls. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* analysis in SPSS software (ver. 25 x86-x64; IBM Corp., Armonk, NY) were conducted to detect significant differences. A value of $p < 0.05$ was considered indicative of statistical significance.

RESULTS

I. Identification of a TCS critical for normal cell division of *B. glumae* BGR1

To identify a key TCS important for normal cell division of *B. glumae* BGR1, I first mutagenized it with mini-Tn*5* and examined the morphology of the mutants. The mutant RT271 formed filamentous cells when grown in LB medium (Fig. 1A), and its respective mutant complementation, RT271C, restored the rod-shaped cells similar to those in the wild-type BGR1 (Fig. 1A). To determine the insertion site of mini-Tn*5* in the RT271 mutant, a mini-Tn*5* insertion along with flanking sequences was rescued by digestion of its genomic DNA with *Eco*RI, self-ligation, and transformation into *E. coli* DH5α. Flanking sequences of mini-Tn*5* from the rescued plasmid pRT271E revealed that an annotated gene BGLU_1G13360 had an insertional mutation (Fig. 1B).

This gene, *gluR*, encoded a 27.7 kDa protein that exhibited 99.6% similarity to known OmpR-type response regulators such as BURPS305_7006 in *B. pseudomallei* 305, RisA (BMA10247_1253) in *B. mallei* NCTC 10247, and BCENMCO3_1962 of *B. cenocepacia* MCO-3 (Appendix 1). Downstream of *gluR* was a putative sensor kinase, *gluS* (BGLU_1G13350), (Fig. 1B) that showed 96.7, 94.1, and 90.0% identities with known sensor kinases such as Envz1

(BGL_1C23830) in *B. plantarii*, BGLA_1G24110 in *B. gladioli* BSR3, and RisS (BMA1486) in *B. mallei* ATCC 23344, respectively (Appendix 1).

Due to the proximity of *gluS* and *gluR* in the BGR1 genome, I reasoned that these two genes might be co-transcribed into a polycistronic RNA. Therefore, I performed reverse transcription-polymerase chain reaction (RT-PCR) with specific primers, GluSR-F/R (Fig. 1B, Table 2). I found that *gluR* and *gluS* were indeed co-transcribed (Fig. 1C). Next, I mutagenized pBGH1, a cosmid carrying *gluS* and *gluR*, with Tn*3-gusA* to generate mutants of *gluR* and *gluS* followed by marker-exchange into *B. glumae* BGR1, resulting in BGLUR133 (BGR1 *gluR*::Tn*3-gusA133*) and BGLUS35 (BGR1 *gluS*::Tn*3-gusA35*) (Fig. 1B). Even though *gluR* and *gluS* were co-transcribed, the insertion of Tn*3-gusA133* in *gluR* did not cause polar effect (Fig. 1d).

II. Aberrant cell division due to a mutation in *gluR*

To determine whether the insertion of Tn*3-gusA* in *gluR* or *gluS* conferred a similar cell morphology to the RT217 mutant, I observed the morphology of the *gluR* and *gluS* mutants under a light microscope. The *gluR* mutant BGLUR133 showed extensive filamentous cells in LB medium (Fig. 2), consistent with the initial phenotype of the *gluR*::min-Tn*5* mutant RT271 in LB (Fig. 1A), however, the *gluS* mutant BGLUS35 formed normal cells in LB medium (Fig. 2). The *gluR*

mutant BGLUR133 maintained a normal rod-shaped cell morphology similar to that of the *gluS* mutant BGLUS35 in M9 minimal medium (Fig. 2).

Transmission electron microscopy (TEM) of ultrathin sections of the *gluR* mutant BGLUR133 revealed characteristic features of filamentous cells with multiple nuclei and indents along the cell membrane at points where the septum would have formed to separate dividing cells (Fig. 3). The genetically complemented strain of the *gluR* mutant with pBGH13, BGLUR133C, had morphologically uniform rod-shaped cells (Fig. 3). The growth of the *gluR* mutant BGLUR133 and the wild-type BGR1 for 30 hours in LB medium at 37^{o}C was similar (Fig. 4A, B). Although filamentous cells of the *gluR* mutant BGLUR133 remained viable for 30 hours, their abundance decreased after 18 hours, as observed under the microscope (Fig. 4A).

III. Direct control of genes involved in cell division by GluR

Because TEM suggested the involvement of GluR in cell division, I determined whether GluR influences the expression of genes in the *dcw* cluster involved in cell division. In *B. glumae*, there were 15 annotated genes, *e.g.*, *ftsA*, *ftsI*, *ftsL*, *ftsQ*, *ftsW*, and *ftsZ* in the *dcw* cluster and *ftsB* and *ftsK* in other regions (Fig. 5A, B). The expression levels of *ftsA*, *ftsB*, *ftsI*, *ftsK*, *ftsL*, *ftsQ*, *ftsW*, and *ftsZ* in the *gluR* mutant BGLUR133 were significantly increased compared to those in the wild-type BGR1

(Fig. 5C). The expression levels of the eight genes in BGLUR133C were similar to those in the wild type (Fig. 5C). To determine whether GluR directly controls their expression, I performed EMSA on the putative promoter regions of *ftsA* and *ftsZ* and purified His-tagged GluR (GluR-His). The binding of GluR-His to the putative promoter regions of *ftsA* and *ftsZ* confirmed that GluR-His directly represses the expression of cell division genes in *B. glumae* (Fig. 5D). A conserved inverted repeat sequence was found in the upstream regions of *ftsA* and *ftsZ*, indicating a potential GluR binding site (Fig. 6).

IV. Alleviation of aberrant cell morphology by constitutive expression of *ftsA* in the *gluR* mutant

Because the FtsA to FtsZ ratio is critical for normal bacterial cell division, I evaluated the role of GluR in its maintenance. Taking the expression levels of *ftsA* and *ftsZ* in the wild type as 1.00, the expression levels of these two genes were 1.21 and 1.67, respectively, in the *gluR* mutant BGLUR133 (Fig. 5C). To confirm that imbalanced expression of *ftsA* and *ftsZ* causes abnormal cell division, *ftsA* was constitutively expressed under the control of the *trc* promoter in the wild type, the *gluR* mutant BGLUR133, and the complemented strain BGLUR133C; the resulting strains were designated BGR1(pFtsA), BGLUR133(pFtsA), and BGLUR133C(pFtsA), respectively. Cells of BGLUR133(pFtsA) showed normal

cell division as well as a 5.2-fold increase in *ftsA* expression (Fig. 7A, B). Moreover, *ftsA* expression was increased more than 100-fold in BGR1(pFtsA) and BGLUR133C(pFtsA), whose cells underwent abnormal division (Fig. 7A, B).

V. Influence of glutamate and glutamine on GluR-mediated control of cell division

Because the *gluR* mutant BGLUR133 formed filamentous cells in LB but not in M9 minimal medium, I reasoned that the amino acids in LB medium might be the cause of filamentous cell formation. Therefore, I added 10% casamino acids to M9 minimal medium containing glucose to evaluate their influence on the morphology of the *gluR* mutant BGLUR133. Adding casamino acids to M9 minimal medium transformed the morphologically normal cells of the *gluR* mutant BGLUR133 into filamentous cells (Fig. 8). To identify the amino acid(s) responsible for triggering filamentous cells in the *gluR* mutant BGLUR133, 20 amino acids were individually added to M9 minimal medium. Of the 20 amino acids, only glutamine and glutamate individually triggered cells of the *gluR* mutant BGLUR133 to become filamentous in M9 minimal medium (Fig. 9A, B). These results suggested glutamine and glutamate play a role in *gluR*-mediated cell division in *B. glumae*.

Because environmental glutamine affected the cell morphology of the *gluR* mutant BGLUR133 in M9 minimal medium, I examined the expression levels of

seven *fts* genes in M9 minimal medium with or without glutamine. In the absence of glutamine, the expression levels of the seven *fts* genes were significantly lower in the *gluR* mutant BGLUR133 than in the wild type, or the BGLUR133C complemented strain (Fig. 9C). However, the addition of glutamine to M9 minimal medium increased the expression levels of the seven *fts* genes in the *gluR* mutant BGLUR133 (Fig. 9D).

VI. Heat sensitivity due to altered *fts* gene expression in the *gluR* mutant

Because *fts* genes were identified in a temperature-sensitive filamenting mutant, I assessed whether the filamenting *gluR* mutant BGLUR133 is heat sensitive. Despite having no effect at 37^{o}C (Fig. 4B), the number of cells of the *gluR* mutant BGLUR133 decreased significantly after 6 hours at 42°C, and they were entirely nonviable after 12 hours in LB medium (Fig. 10A). The wild-type BGR1, the *gluS* mutant BGLUS35, and the complemented strain BGLUR133C showed no growth but prolonged survival at 42°C (Fig. 10A). In M9 minimal medium at 42°C, the *gluR* mutant BGLUR133 retained viability for 18 hours and subsequently lost viability (Fig. 10B). By contrast, the wild-type BGR1 and the complemented strain BGLUR133C increased in cell number during the static period of *gluR* mutant BGLUR133 in M9 medium (Fig. 10B).

DISCUSSION

In addition to QS systems, pathogens also respond to environmental factors using TCSs. Here, I investigated the response regulator, GluR, which is crucial for normal cell division in *B. glumae*. Although *gluR* and *gluS* were co-transcribed, GluS may not be a bona fide counterpart of GluR because a mutation in *gluS* did not affect the normal cell division of *B. glumae*. Such a genetically linked but functionally independent TCS system was reported for *risS* and *risA*, which encode a sensor kinase and a response regulator, respectively, in *B. pertussisi* (Stenson *et al*., 2005). *risS* and *risA* were genetically linked but functionally independent (Stenson *et al*., 2005). Phosphorylation of RisA was mediated by crosstalk with a non-operonic histidine kinase, RisK (Chen *et al*., 2017). Therefore, an as-yet-unidentified sensor kinase may be responsible for the phosphorylation of GluR in *B. glumae*.

In rod-shaped bacteria such as *B. glumae*, cell division involves ingrowth of the cell wall and membrane, forming a septum between two replicated chromosomes (Harry, 2001). To ensure equal partitioning of chromosomes into daughter cells, the expression of genes involved in cell division must be properly regulated (Dai and Lutkenhaus, 1992; Harry, 2001). In most bacteria, cell division and cell-wall synthesis are regulated by a series of genes in the *dcw* cluster (Dai and Lutkenhaus, 1992). Within bacterial groups of the same class and cell shape, the order and

regulation of genes in the *dcw* cluster are highly conserved (Pilhofer *et al*., 2008). Therefore, it was not surprising that in *B. glumae*, the *dcw* cluster displayed significant similarities to that of *E. coli* (Vicente *et al*., 1998). Pioneer studies of the *dcw* cluster genes in *E. coli* spotlighted *ftsZ* as the key element in cell division (Bi and Lutkenhaus, 1991; de Boer *et al*., 1992). It was later noted that FtsZ is not sufficient to drive septation, leading to the discovery of, for instance, *ftsA*, *ftsQ*, and *ftsI* (Vicente *et al*., 1998). In a hierarchical order initiated by the assembly of FtsZ at the division site, the *dcw* proteins are coordinately involved in cell division and the synthesis of the peptidoglycan precursors (Vicente *et al*., 1998; Lutkenhaus and Du, 2017).

The mechanisms of regulation of the *dcw* cluster are unclear, despite the presence therein of several regulatory elements, *e.g*., internal promoters, transcript stabilizers, and protein ratios (Vicente *et al*., 1998; Francis *et al*., 2000). Studies on the control of cell division have concentrated on FtsZ. Multiple promoter regions have been reported upstream of *ftsZ* in the *dcw* cluster, indicating regulation at the transcriptional level (Vicente *et al*., 1998; Francis *et al*., 2000; Margolin, 2000). I found that GluR binds to the upstream promoter regions of *ftsZ* and *ftsA* located in the *ftsA* and *ftsQ* coding regions, respectively. A conserved inverted repeat sequence was found in the upstream sequences of *ftsA* and *ftsZ*. This suggested a possible GluR binding sequence, albeit more research is needed to validate. Unlike positive regulators in *E. coli*, such as SdiA (Sitnikov *et al*., 1996), the phase-specific

sigma factor (Ballesteros *et al*., 1998), and RcsB (Carballès *et al*., 1999), GluR negatively regulates cell division in *B. glumae*.

In the *dcw* cluster, biased expression of genes resulting from a mutation in *gluR* induced aberrant cell division. The GluR-controlled expression of *dcw* cluster genes was essential for normal cell division in *B. glumae*. FtsZ may be tethered to the membrane by two cytoplasmic membrane-associated proteins, ZipA and FtsA (Pichoff and Lutkenhaus, 2005), thus mediating cell division. Each *E. coli* cell is estimated to contain 3000–5000 molecules of FtsZ, 50–200 of FtsA, and 1500 of ZipA (Ortiz *et al*., 2016). In addition, an imbalanced FtsZ and FtsA ratio is detrimental to *E. coli* (Dai and Lutkenhaus, 1992; Tamura *et al*. 2006)). In the filamentous cell-forming *gluR* mutant, expression of *ftsZ* was higher than that of *ftsA*, but the constitutive expression of *ftsA* restored a normal morphology. Although the optimal FtsZ and FtsA ratio is unknown, a 5.2-fold increase in *ftsA* expression in the *gluR* mutant restored the normal rod-shaped cell phenotype.

Because LB medium is rich in amino acids, and filamentation of the *gluR* mutant was facilitated by supplementation of extracellular glutamine or glutamate in M9 medium, the glutamine-dependent filamentous cell formation at an early stage of growth in LB was explicable. However, the number of filamentous cells of the *gluR* mutant BGLUR133 decreased over time, possibly because of depletion of amino acids, including glutamine and glutamate, 12 hours after incubation (Fig. 11**)**. Since the *gluR* mutant formed filamentous cells in glutamine or glutamate-dependent

manner, I hypothesized that GluR phosphorylation is caused by extracellular glutamine or glutamate, which promotes proper cell division by repressing *dcw* cluster genes. Extracellular glutamine and glutamate reportedly alter the expression of genes involved in cell division and cell-wall synthesis of *B. subtilis* (Ye *et al*., 2009). Beuria *et al*. reported an increased FtsZ polymerization rate and extent in *E. coli* resulted from extracellular glutamine (Beuria *et al*., 2003). It was noted that FtsZ showed optimal polymerization as large, bundled filamentous structures in *E. coli* in the presence of 1 M glutamine (Beuria *et al*., 2003). Interestingly, FtsZ polymers formed in the absence of glutamine were ninefold less stable than those in its presence, emphasizing the roles of these amino acids in the stability of FtsZ polymers (Beuria *et al*., 2003).

Connections between TCS and glutamine metabolism have been reported in other bacteria. For example, GlnK-GlnL of *Bacillus subtlis* (Satomura *et al*., 2005), GluR-GluK of *Streptomyces coelicolor* (Li *et al*., 2017), and AauR-AauS of *Pseudomonas putida* (Sonawane *et al*., 2006) reportedly sense and control glutamate uptake. In other bacteria, the TCSs involved in glutamine sensing and uptake are located close to the glutamine ABC transporter (Satomura *et al*., 2005; Sonawane *et al*., 2006; Li *et al*., 2017). However, GluR is not likely to be involved in glutamine uptake because, our group previously reported that GltI is responsible for glutamine uptake in *B. glumae* (Kang *et al*., 2017). A *bona fide* sensor kinase

responsible for glutamine sensing and GluR phosphorylation is yet to be identified in *B. glumae*.

Physiological experiments in *E. coli* demonstrated that mutated septation genes resulted in elongated cells and an exponential population decrease at high temperatures, giving the mutants the name filamentous temperature sensitive (Ricard and Hirota, 1973). While I did not specifically modify the septation genes of *B. glumae* in the *gluR* mutant BGLUR133, I observed identical phenotypes of cell elongation and sensitivity to heat treatment at 42°C as in *E. coli* with mutated septation genes (Ricard and Hirota, 1973). These findings further support the hypothesis that GluR is crucial for cell division and an optimum gene expression profile. Taken together, my findings indicate that GluR is key for maintaining the gene expression profile required for glutamine- or glutamate-dependent control of cell division in *B. glumae* BGR1.

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Table 1. Bacterial strains

Strain or plasmid	Characteristics	Source
Burkholderia glumae		
BGR1	Wild type, Rif^R	Kim *et al*., 2004
BGLUR133	BGR1 *gluR*::Tn*3-gusA133*, Km^R	This study
BGLUS35	BGR1 *gluS*::Tn*3-gusA35*, Km^R	This study
BGLUR133C	BGR1, *gluR*::Tn*3-gusA133* containing pBGH13, Km^R, Tet^R	This study
BGLUS35C	BGR1, *gluS*::Tn*3-gus35* containing pBGH13, Km^R, Tet^R	This study
RT271	BGR1 *gluR*::mini-Tn*5rescue*, Km^R	This study
RT271C	BGR1 *gluR*::mini-Tn*5rescue* containing pBGH1, Km^R, Tet^R	This study
Escherichia coli		
DH5α	F^- Φ80d*lacZ ΔM15*(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* ($r_k^+m_k^+$ *supE44 thi-1 gyrA relA1*	Gibco BRL
BL21 (DE3)	F^- *ompT hsdS*$_B$ ($rB^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmid		
pBluescript II SK (+)	Cloning vehicle; phagemid, pUC derivative, Amp^R	Stratagene
pRK2013	Tra^+, ColE1 replicon, Km^R	Figurski and Helinski, 1979
pLAFR3	Tra^-, Mob^+ RK2 replicon, Tet^R	Staskawicz et al., 1987
pBGH1	13 kb BGR1 library clone containing *gluS* and *gluR* cloned into pLAFR3, Tet^R	This study

pBGH13	6.3 kb *Bam*H1-*Hin*dIII fragment containing *gluS* and *gluR* gene cloned into pLAFR3, Tet^R	This study
pET21b	T7 promoter-based expression vector, Amp^R	Novagen
pGluR-His	*gluR* in pRT21b, Amp^R	This study

Rif^R, rifampicin resistance; Tet^R, tetracycline resistance; Km^R, Kanamycin resistance; Amp^R, ampicillin resistance; Sp^R, Spectinomycin resistance.

Table 2. List of primers.

Primers	**Sequence (5' to 3')**
GluSR-F	CAGCCCGCGCTTCATTCAGA
GluSR-R	TGACCGCGATCAGCAGCGCG
FtsA-F	CTGCAAGATCACCAACGTTT
FtsA-R	TCGGTCTGCGTGACCTCCTT
FtsB-F	AGGATTTGCAGAACGGCACC
FtsB-R	ATTCGGCGAAACGAAACTGC
FtsI-F	ACATCACCTACGCCAACCRG
FtsI-R	CGGGTAATTGACGAGCGAGA
FtsK-F	GCATGTGGTCGCTGAAGGT
FtsK-R	GGAAGTACAGCGACAGACCG
FtsL-F	ATCTTCTTCCAGTTGCAGCGT
FtsL-R	CTCGATGCGCGAGGTCTT
FtsQ-F	AGGGCAACTTCTTCACGGTC
FtsQ-R	GAGCGGCTTGTATTCCTCCA
FtsW-F	CGTGGGACGAGCGCTATGCG
FtsW-R	TAGTTGAGCTTCTCGACGCT
FtsZ-F	ATGCCGAGATGGACAAGTGC
FtsZ-R	TCGAAGTCGACGTTAACGAG
16S RNA-F	AGCCGCGGTAATACGTAGG
16S RNA-R	ACTCTAGCCTGCCAGTCACC

KatE1-F	ACTCGCGCCGCTCGTCGAA
KatE1-R	ACATCGGCATCCTGGGTCGC
gluR_Nde1-F	GGCATATGATGCCGCCCATGGAAACTAA
gluR_BamH1-R	CCGGATCCGTCCGGGATGAAGACGTAGC
ftsAp- F	CGTCGCGCTACGCCTGGACG
ftsAp- R	TGTCGCTCAGAAAGCGCATG
ftsZp- F	TCGACGGCGATGGGCCTGCT
ftsZp- R	AAAGATATAACCACTCGGCC

F, forward; R, reverse.

Fig. 1. The GluS-GluR TCS of *B. glumae* BGR1. **(A**) Microscopic observation of the cell morphology of BGR1 (wild type), RT271 (BGR1, *gluR*::mini-Tn*5rescue*), and mutant complemented RT271C (BGR1, *gluR*::mini-Tn*5rescue* carrying pBGH1) strains in LB medium. (**B**) Organization scheme of the GluS sensor kinase and GluR response regulator in pBGH1. Vertical bars above the restriction map indicate the position of the Tn*5* (271) and Tn*3-gusA* insertions (35 and 133). E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sac*I. The map below pBGH1 represents pBGH13 plasmid of a 6.3 kb fragment containing *gluS* and *gluR* gene**.** **(C**) RT-PCR analysis showing that *gluS* and *gluR* genes are co-transcribed in BGR1. Primers were designed to amplify a 237 bp (*) product encompassing the *gluR* and *gluS* genes in the wild type. Lane G, PCR product using genomic DNA as a template; Lane R, PCR product using RNA as a template; Lane C, PCR product using cDNA as a template. (**D**) No polar effect resulting from Tn*3-gusA* insertion. Lane bp, marker; Lane G1, PCR product from *gluR* chromosomal DNA as a template; Lane G2, PCR product from *gluS* chromosomal DNA as a template; Lane R, PCR product from total RNA as a template; Lane C1, PCR product from *gluR* cDNA as a template; Lane C2, PCR product from *gluS* cDNA as a template. The thick bars below the gene map indicate the position that was amplified for cDNA synthesis. Primers were designed to amplify 200 bp (1), and 216 bp (2) product of *gluS* and *gluR* respectively.

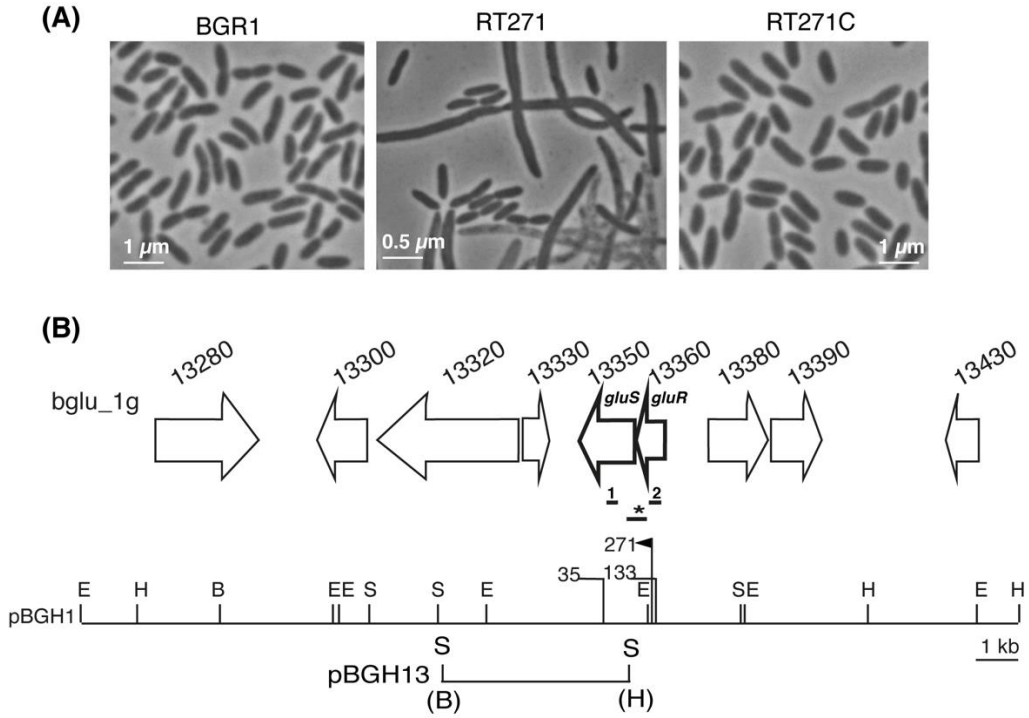

(A)
BGR1
RT271
RT271C
1 μm
0.5 μm
1 μm
(B)
bglu_1g
13280
13300
13320
13330
13350
13360
13380
13390
13430
gluS
gluR
1
2
271
35
133
E H B EE S S E E SE H E H
pBGH1
1 kb
S S
pBGH13
(B) (H)
(C)
237 bp
bp G R C
300
200
100
(D)
BGR1
(Wild type)
bp G1 G2 R C1 C2
300
200
100
BGR1
(gluR::Tn3-gusA133)
bp G1 R C1 C2
300
200
100
BGR1
(gluS::Tn3-gusA35)
bp G2 R C1 C2
300
200
100

Fig. 2. Tn*3*-*gusA* mutations in *gluR* resulted in nutrient-dependent cell filamentation. In LB medium, the *gluR* mutant BGLUR133 formed filamentous cells, but a normal rod-shaped cell morphology was observed in M9 minimal medium. No morphological defects were observed in the *gluS* mutant BGLUS35 in the different culture media.

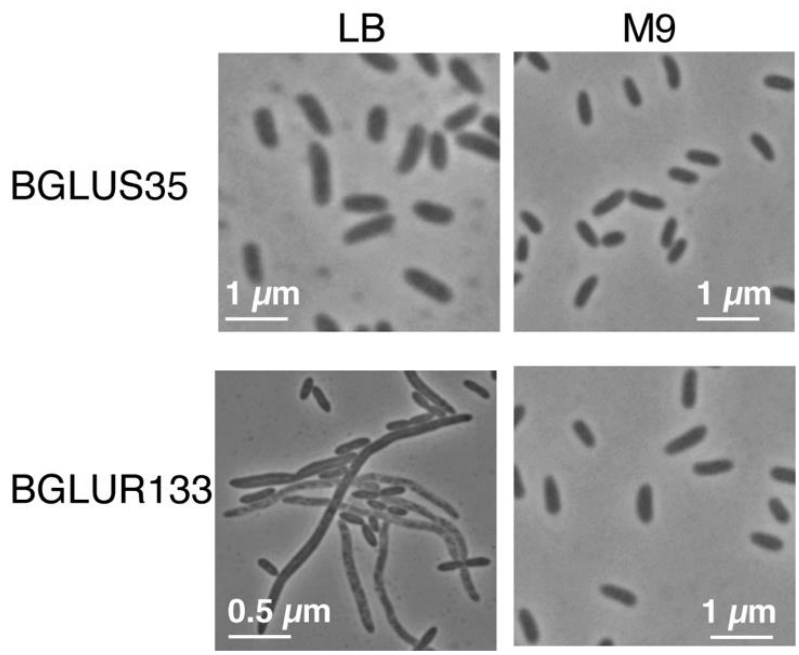


Fig. 3. The *gluR* mutant forms a heterogeneous population of viable filamentous and normal rod-shaped cells. The indicated bacterial strains were grown to the early stationary phase, and the morphological phenotypes of ultrathin sections were observed by TEM. BGLUR133-M shows that the filamentous cells formed by the *gluR* mutant contained multiple nuclei (arrows) with indents (arrowhead) along the cell wall, symbolizing failed septum formation.

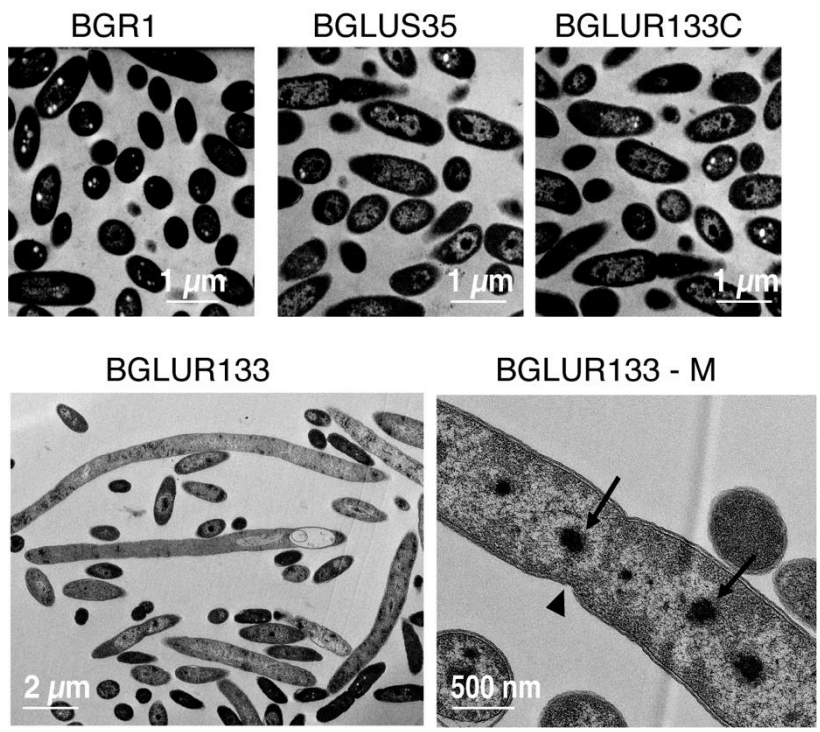
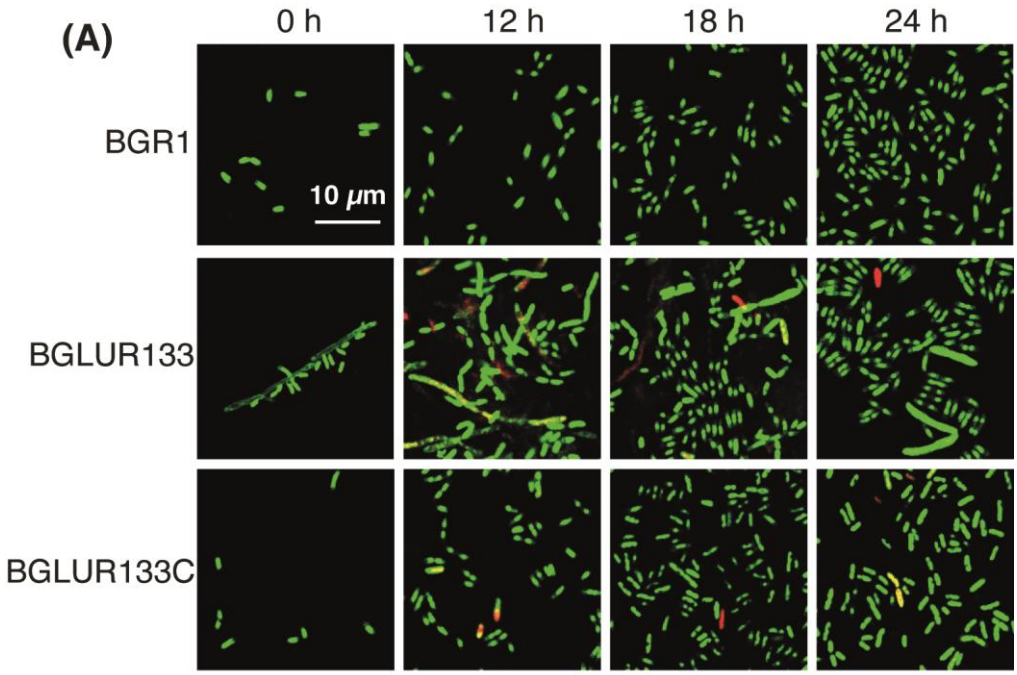


Fig. 4. The role of GluR on the growth and viability of *B. glumae*. (**A**) Cell viability of the wild type, the *gluR* mutant BGLUR133, and complemented BGLUR133C strains assessed by combination staining with propidium iodide (PI) and SYTO 9 green. Fluorescence images were obtained by confocal laser scanning microscopy. Dead cells stained with PI are red, and SYTO 9-stained viable cells are green. (**B**) Cell population densities of bacteria strains in LB medium were quantified in terms of colony-forming units (CFUs), and the results expressed as means (log CFU/ml) ± standard error (SE) of triplicate results.


(A)
0 h
12 h
18 h
24 h
BGR1
10 μm
BGLUR133
BGLUR133C

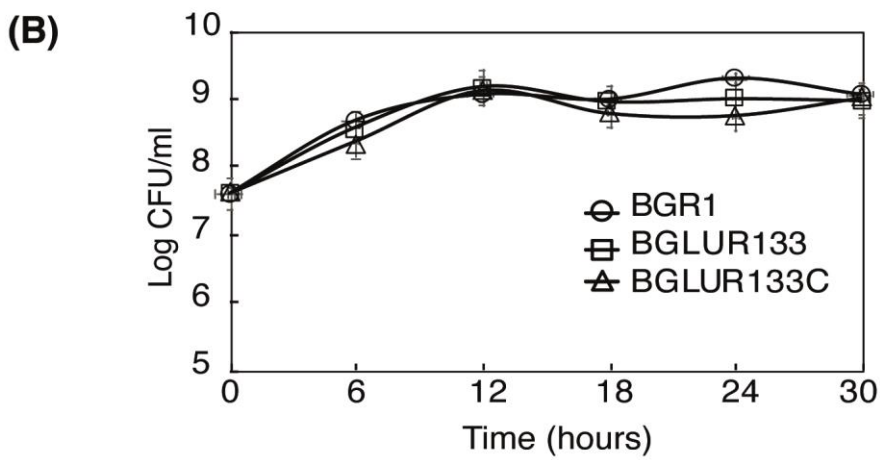

(B)
Log CFU/ml
10
9
8
7
6
5
0
6
12
18
24
30
Time (hours)
BGR1
BGLUR133
BGLUR133C

Fig. 5. GluR represses cell division and septation genes in *B. glumae*. (A) Genetic organization of the *dcw* cluster in BGR1. Dark arrows represent genes involved in septation during cell division, and light arrows are genes involved in cell-wall synthesis or with no known function. The section below the gene map indicates the positions and size of the respective putative promoters in this study. **(B)** Gene maps of additional cell-division genes outside the *dcw* cluster. **(C)** Expression levels of eight cell-division genes in *B, glumae* strains compared by qRT-PCR. mRNA levels were normalized to 16S *rRNA*, and the fold expressions are relative to those of the wild type. Data are means ± standard error (SE) of triplicates; statistical analysis was performed by one-way ANOVA/Tukey's correlation for multiple comparisons. *, $p < 0.05$; ($F(48,71) = 1536.273$; $p = 0.00$). **(D)** Electrophoretic mobility shift assay (EMSA) showed direct control of *ftsA* and *ftsZ* by GluR-His binding to the respective putative promoter regions; 0.75 μM GluR-His, 1 nM labeled target DNA, 1 nM unlabeled *katE* non-competitor DNA, and 20 nM unlabeled target promoter DNA were used for EMSA.

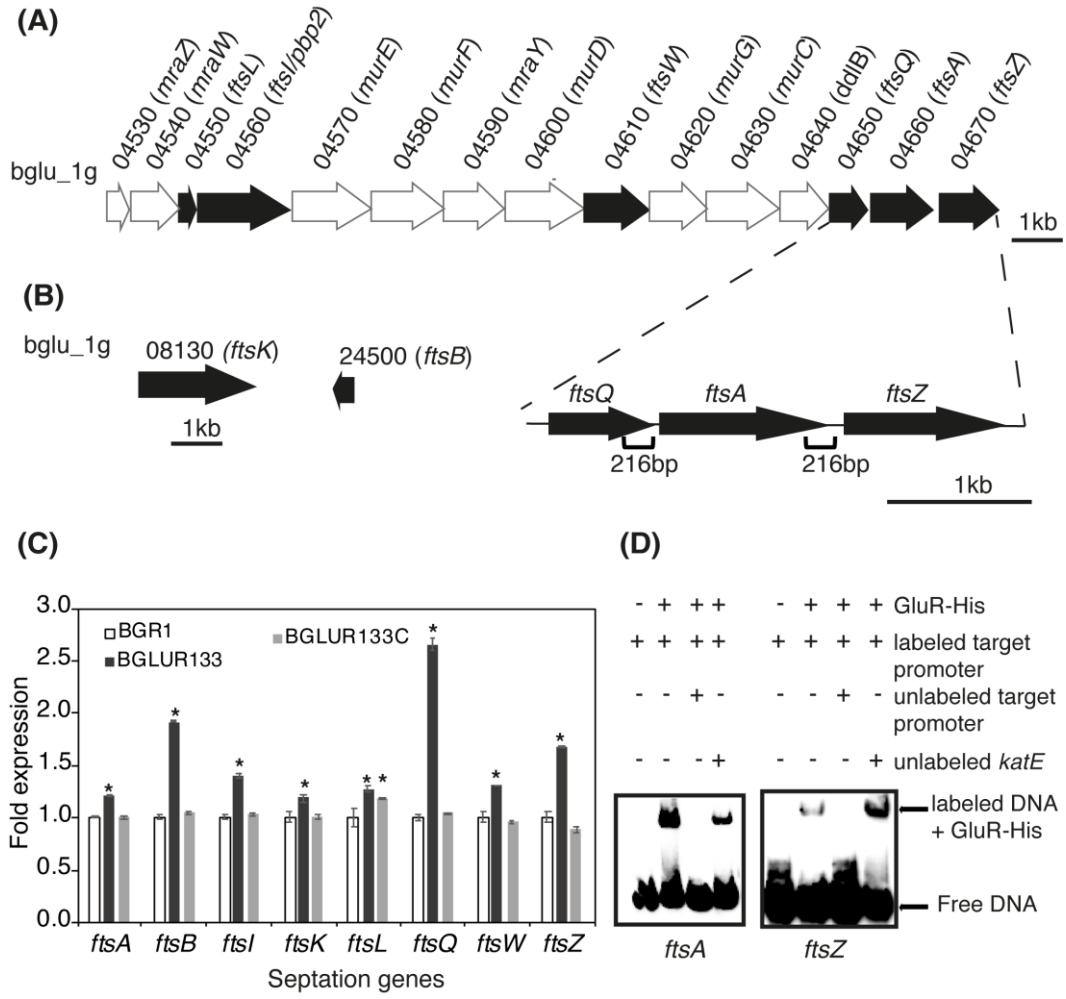

(A)
bglu_1g
04530 (mraZ)
04540 (mraW)
04550 (ftsL)
04560 (ftsI/pbp2)
04570 (murE)
04580 (murF)
04590 (mraY)
04600 (murD)
04610 (ftsW)
04620 (murG)
04630 (murC)
04640 (ddlB)
04650 (ftsQ)
04660 (ftsA)
04670 (ftsZ)
1kb
(B)
bglu_1g
08130 (ftsK)
24500 (ftsB)
1kb
ftsQ
ftsA
ftsZ
216bp
216bp
1kb
(C)
BGR1
BGLUR133
BGLUR133C
Fold expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ftsA
ftsB
ftsI
ftsK
ftsL
ftsQ
ftsW
ftsZ
Septation genes
(D)
- + + + - + + + GluR-His
+ + + + + + + + labeled target promoter
- - + - - - + - unlabeled target promoter
- - - + - - - + unlabeled katE
labeled DNA + GluR-His
Free DNA
ftsA
ftsZ

Fig. 6. **Putative GluR binding site in *ftA* and *ftsZ* gene upstream sequences.** The conserved sequences present in upstream regions of *ftsA* and *ftsZ* are highlighted. The inverted repeat sequences are indicated by the arrows.

Fig. 7. GluR maintains the molar ratio of *ftsZ* to *ftsA* to ensure normal cell division. *FtsA* was constitutively expressed to counteract *ftsZ* in the wild type (BGR1), BGLUR133, and BGLUR133C strains. (**A**) mRNA levels were quantified by qRT-PCR and are shown as normalized fold expression values. Data are means ± standard error (SE) of triplicates. (**B)** Bacterial strains cultured to early stationary phase were visualized using a Carl Zeiss GmbH Auriga microscope. pFtsA represents constitutive expression of *ftsA* in the indicated bacterial strains.

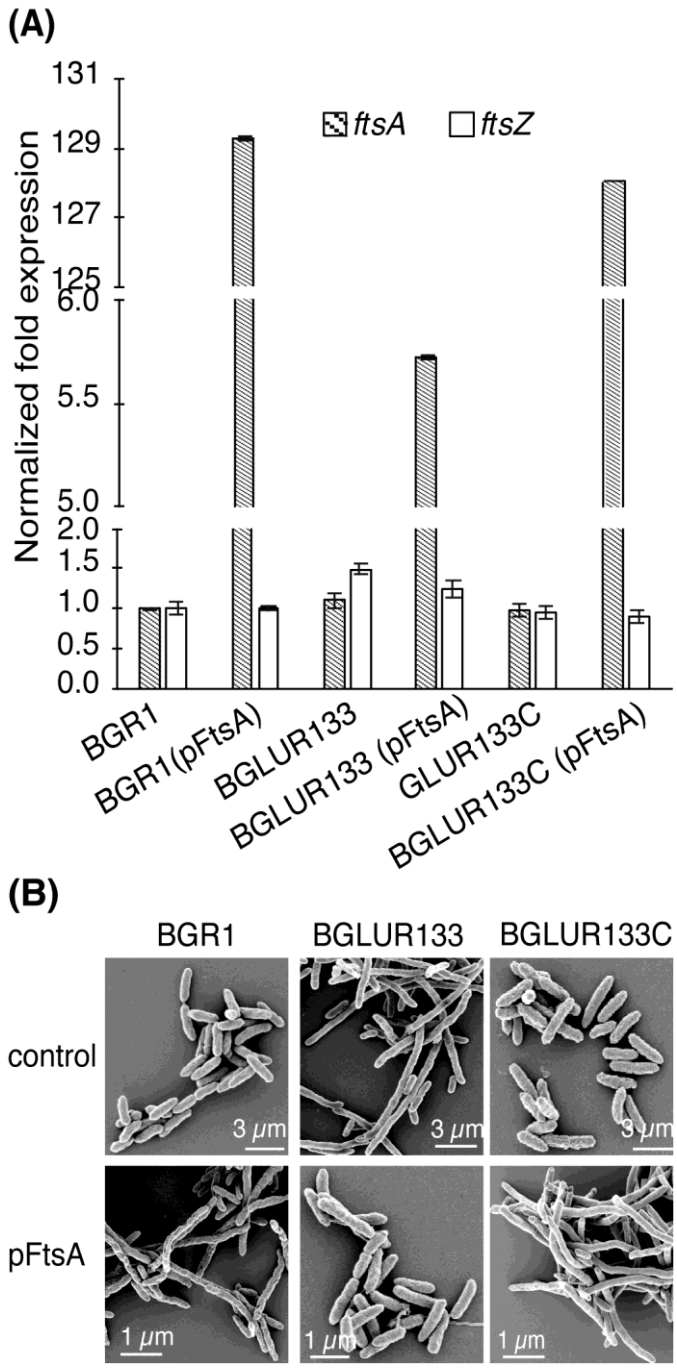

(A)
Normalized fold expression
131
129
127
125
6.0
5.5
5.0
2.0
1.5
1.0
0.5
0.0
ftsA
ftsZ
BGR1
BGR1(pFtsA)
BGLUR133
BGLUR133 (pFtsA)
GLUR133C
BGLUR133C (pFtsA)
(B)
BGR1
BGLUR133
BGLUR133C
control
pFtsA
3 μm
1 μm

Fig. 8. Extracellular amino acids promote filamentation in response to GluR mutation. The indicated bacterial strains were cultured overnight in M9 minimal medium with or without 10% casamino acids (CA), processed for SEM analysis, and their morphology observed using a Carl Zeiss GmbH Auriga microscope.

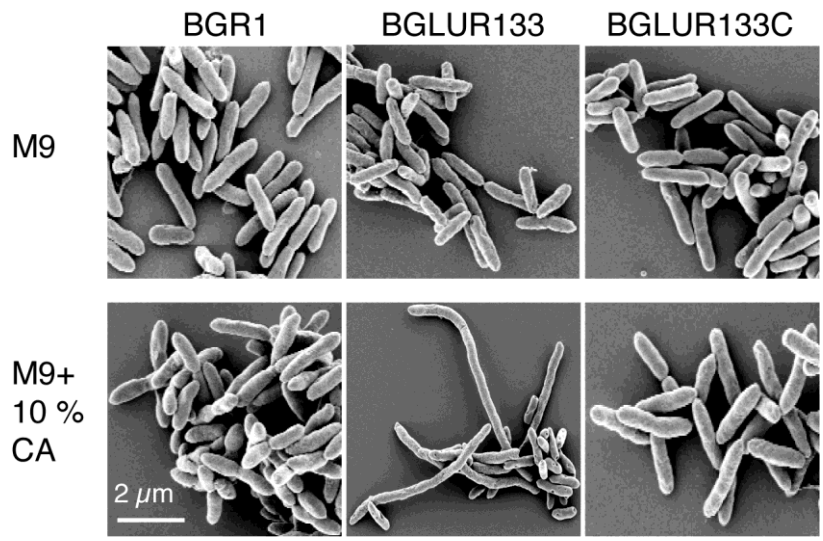


Fig. 9. Extracellular glutamine and glutamate are required for GluR-mediated cell division. The *gluR* mutant BGLUR133 formed filamentous cells in glutamine (**A**) and glutamate (**B**) -rich M9 minimal medium. (**C-D**) Differences in the expression levels of septation genes in plain M9 (**C**) and glutamine-rich M9 minimal medium (**D**), analyzed by qRT-PCR using the wild-type BGR1 as the baseline. Data are means ± standard error (SE) of triplicates.

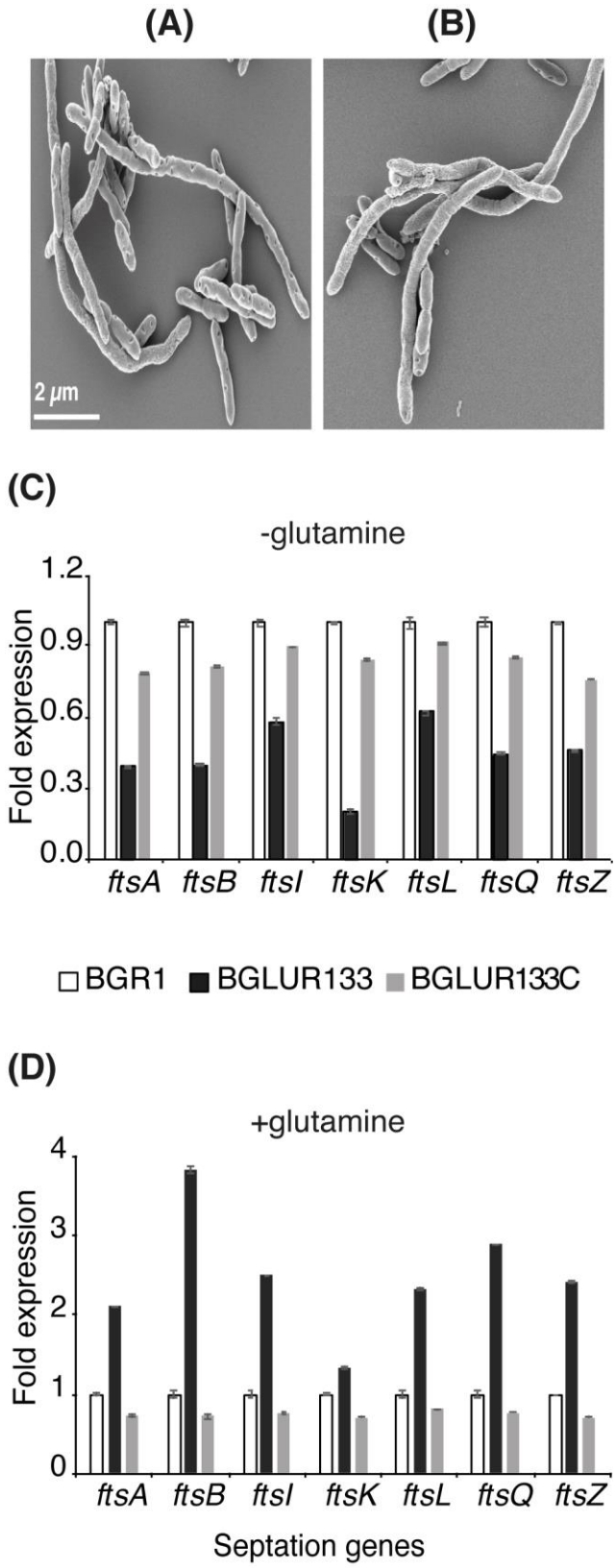

(A)
(B)
2 μm
(C)
-glutamine
Fold expression
1.2
0.9
0.6
0.3
0.0
ftsA ftsB ftsI ftsK ftsL ftsQ ftsZ
BGR1 BGLUR133 BGLUR133C
(D)
+glutamine
Fold expression
4
3
2
1
0
ftsA ftsB ftsI ftsK ftsL ftsQ ftsZ
Septation genes

Fig. 10. Exponential population decline at 42°C as a result of mutations in GluR. At 6-hour intervals, the indicated strains' population densities in LB medium **(A**) and M9 medium (**B**) were quantified by CFU counting, and the results expressed as log CFU/ml. Data are means ± standard error (SE) of triplicates.

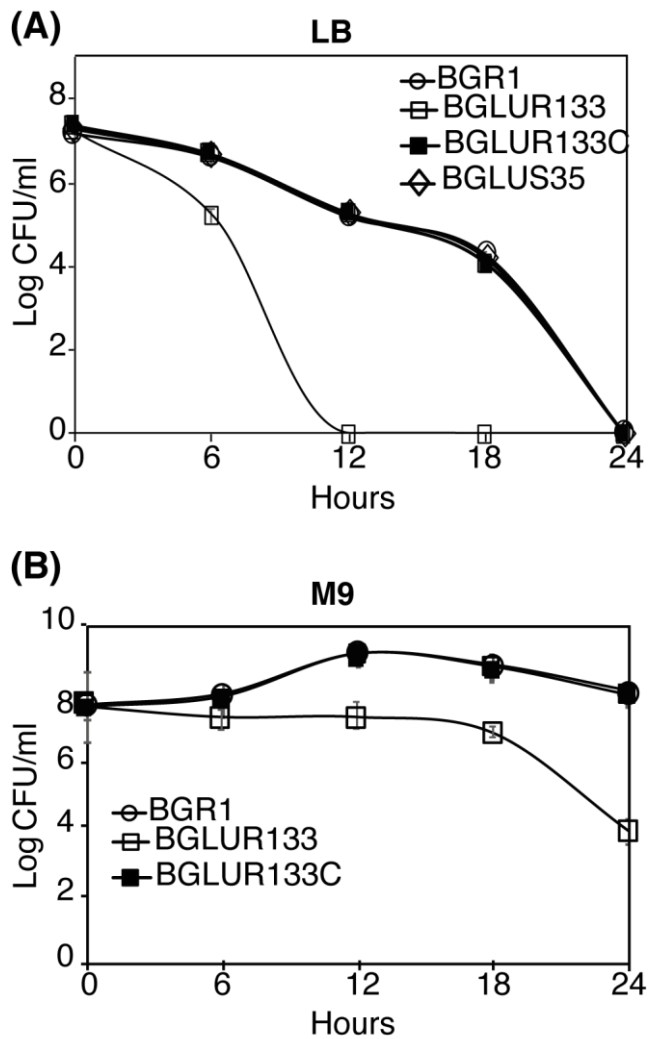
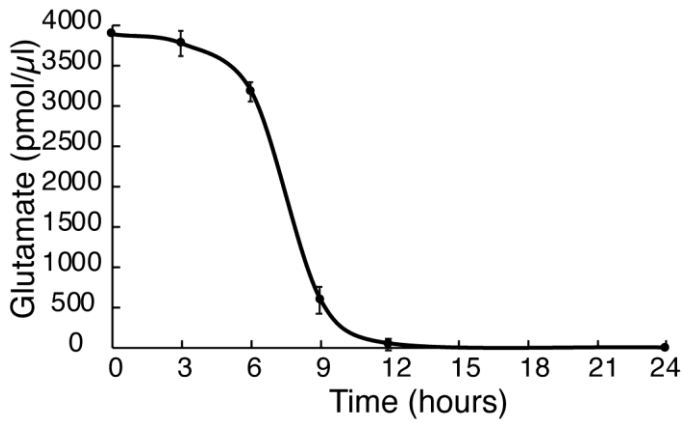


Fig. 11. **Glutamate utilization of *B. glumae* BGR1 in LB culture medium over time.** Data represents the mean ± standard deviation (SD) of triplicates.



CHAPTER 2

Mutations in the Two-Component GluS-GluR Regulatory System Confer Resistance to β-lactam Antibiotics in *Burkholderia glumae*

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Marunga, J., Goo, E., Kang, Y., Hwang, I. (2021).

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ABSTRACT

Given intense selective pressures such as antibiotics, it is not surprising that bacteria have devised sophisticated signaling systems to elicit adaptive responses that override their toxic effects. The two-component system (TCS) is known to play an important role in developing antibiotic resistance. Using the rice pathogen *Burkholderia glumae* BGR1 as a model organism, I showed that the GluS-GluR TCS, a sensor kinase and response regulator, respectively, contributes to β-lactam resistance, but through a distinct mechanism. The inactivation of *gluS* or *gluR* conferred resistance against β-lactam antibiotics in *B. glumae,* whereas the wild type was susceptible to those antibiotics. In *gluS* or *gluR* mutants, expression of genes encoding metallo-β-lactamases (MBLs) and penicillin-binding proteins (PBPs) was significantly increased compared to those in the wild type. GluR-His bound to the putative promoter regions of annotated genes encoding, MBL (BGLU_1G21360) and PBPs (BGLU_1G13280 and BGLU_1G04560), to function as a repressor. These results showed that the potential to attain β-lactam resistance could be genetically concealed in TCS in contrast to the widely accepted view of the role of TCS in antibiotic resistance. My findings introduce a new perspective on the understanding of antibiotic resistance mechanisms and possibly suggest a different therapeutic approach for the successful control of bacterial pathogens.

INTRODUCTION

Irrational use of antibiotics contributes to the emergence of antibiotic resistant pathogens, thus foster the re-occurrence of disease outbreaks. Antibiotic resistance has been linked to complex bacterial systems, such as quorum sensing (Pumbwe *et al*., 2008; Wang *et al*., 2019), extra-cytoplasmic functions (ECF), sigma factors (Yoo *et al*., 2016; Woods and McBride, 2017), and two-component systems (TCSs) (Gooderham and Hancock, 2009; Tierney and Rather, 2019). TCSs characterized by sensor kinases and response regulators are crucial for survival and adaptability in each environment (Hoch, 2000; Miller *et al*., 2004; Lingzhi *et al*., 2018). Interest in their roles in antibiotic resistance has recently increased, creating potential targets for new treatments. TCSs are known to direct the process of antibiotic resistance leading to drug target modification, decreased influx, increased outflow, regulation of antibiotic degrading enzymes, biofilm formation, and stress induction (Tierney and Rather, 2019). Examples of TCSs reported to regulate antibiotic resistance include PhoP-PhoQ of *Pseudomonas aeruginosa* that triggers resistance to polymyxin B (McPhee *et al*., 2006; Gooderham and Hancock, 2009); VanS-VanR of *Enterococcus faecium*, and *Streptomyces coelicolor* reduces the affinity to vancomycin (Arthur *et al*., 1992; Hong *et al*., 2008); CreB-CreC of *Escherichia coli* and VbrK-VbrR of *Vibrio parahaemolyticus* triggers β-lactam resistance through the expression of β-lactamases (Zamorano *et al*., 2014; Li *et al*., 2016). For

the case of *Burkholderia glumae*, a notorious pathogen that causes rice panicle blight (Kim *et al*., 2004; Goo *et al*., 2015), there are limited reports on the pathogen's resistance mechanisms against antibiotics. A few studies reported the emergence of oxolinic acid-resistant strains (Hikichi *et al*., 1998; Maeda *et al*., 2004) and a possibility of multi-drug resistance to kanamycin and ampicillin in *B. glumae* (Zhou-qi *et al*., 2016).

The paradigm shifts in resistance and the repeated outbreaks of disease indicate that much remains to be learned about bacterial mechanistic approaches staged to bypass lethal antibiotic effects. Therefore, in this study, I sought out TCSs of *B. glumae* BGR1 and the possible roles in antibiotic resistance. In chapter 1, I demonstrated that the GluR response regulator, rather than its analogous sensor kinase, GluS, regulates cell division. Adding to the reasoning that this TCS pair may serve as therapeutic targets or become a potential trigger of the resistance phenotype, in this chapter, I report that the GluS-GluR TCS of *B. glumae* BGR1 is a functional pair by default negatively regulating the expression of metallo-β-lactamases (MBLs) and β-lactam target sites—penicillin-binding proteins (PBPs). My findings show a remarkable degree of plasticity in this important TCS and add the notion that bacteria can lurk in potential therapeutic targets and store preliminary mechanisms for drug resistance.

MATERIALS AND METHODS

I. Bacterial strains and growth conditions

The bacterial strains, plasmids, antibiotics, and the growth conditions used in this chapter are described in chapter 1 Materials and Methods section I.

II. β-lactam susceptibility test

A cell volume of 4×10^8 cell/ml from overnight Luria Bertani (LB) cultures was grown to mid-log phase in fresh LB broth followed by spreading an equal volume of cells on LB agar supplemented with β-lactam antibiotics in varying concentrations. Bacterial growth was quantified by direct counting using a colony-forming unit (CFU) method using LB agar containing no antibiotics. β-lactam antibiotics and concentrations used: penicillin-G, ampicillin, and carbenicillin at 25, 50, 75, 100, and 150 μg/ml.

III. Viability assay

To supplement the susceptibility test, I performed a live and dead cell assay on the bacterial cells exposed to 50 μg/ml of carbenicillin antibiotics for 0, 12, 24, and 36 h using LIVE/DEAD BacLight bacteria viability kit containing SYTO 9 green-

fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide as described in Chapter 1 (Materials and Methods, section IV).

IV. β-lactamase activity assay

β-lactamase activity was quantified by the hydrolysis of a chromogenic substrate, nitrocefin (DAWIN$_{BIO}$, Abcam, USA), as previously described (Li *et al*., 2016) with a few modifications. Overnight LB cultures were sub-cultured into LB containing 50 μg/ml of carbenicillin for 36 hours. At 12-hour intervals, cells were harvested, washed, and dissolved in phosphate-buffered saline (PBS, pH 7.4), and thereafter sonicated using SONICS vibra Cell (Sonics & Materials INC. Newtown, CT, USA). The cell lysate was incubated with 50 μg/ml of nitrocefin for 10 minutes at room temperature, and OD_{450} was measured.

V. Detection of penicillin-binding proteins

Bacterial membrane harboring PBPs were prepared from LB-carbenicillin (50 μg/ml) cultures grown for 12 hours. Uninhibited PBPs were labeled with fluorescent penicillin, Bocillin FL (Boc- FL, Invitrogen, USA), and quantified by measuring fluorescence intensity as previously described (Zhao *et al*., 1999). To observe PBP localization in the bacterial membrane, Boc-FL labeled cells were

further subjected to a membrane dye, FM-6-64 (Invitrogen, USA) (Kocaoglu *et al*., 2012) and thereafter observed using a confocal laser scanning microscope (Leica SP8X, Wetzla, Germany) at excitation/emission wavelength 488/500-535 and 515/621-678 nm for Boc-FL and FM-6-64 dyes respectively.

VI. Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from *B. glumae* strains and cDNA was synthesized as described in chapter 1(Material and Method section VI). Quantitative PCR was performed using primer sets listed in Table 1**,** bmlA-F/R, MBL1-F/R, MBL2-F/R, and AmpC-F/R to amplify-β-lactamases: Class A, Metallo-β-lactamases, and class C respectively, and PBP1A-F/R, PBP1 -F/R, PBP2A – F/R, PBP2 -F/R to amplify PBPs: PBP1A, PBP1, PBP2A, and membrane carboxypeptidase, respectively. Transcription levels were determined using SsoFast EvaGreen Supermix as described in chapter 1.

VII. Electrophoretic mobility shift assay (EMSA)

I used purified GluR-His from chapter 1. Using primer sets 1g21360p-F/R, 1g13280p-F/R, and 1g04560p-F/R listed in Table 1, the respective promoter regions of the putative GluR targets DNA were amplified. The resulting PCR products were labeled as described in chapter 1(Methods and Material section XII).

329 bp upstream of *katE1* was used as a non-specific competitor DNA amplified using the KatE1-F/R primers (chapter 1, Table 2). The purified GluR-His (1-2 µM) was incubated in binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% (v/v) glycerol) containing 2 nM of biotin-labeled DNA following previously described procedures (Kim *et al*., 2007) and the competion assay carried out as described in chapter 1 (Material and methods section XII).

VIII. Statistical analysis

Statistical analysis was performed as described in chapter 1 (Material and Methods, section XIII). A p-value < 0.05, 0.005, and 0.0005 to indicate statistical significance was used as indicated.

RESULTS

I. Mutations in GluS-GluR TCS associated with β-lactam antibiotic resistance in *B. glumae*

Assessing the response of the wild-type BGR1 and *gluS* and *gluR* mutants to treatment with carbenicillin revealed that while the wild type succumbed to the lethal effects of carbenicillin as shown by the rapid cell death, the *gluS* mutant BGLUS35 and *gluR* mutant BGLUR133 strains sustained growth (Fig. 1A). In the antibiotic environment, both mutants required approximately 12 hours of acclimatization, after which they gained momentum to achieve growth and multiplied to similar levels as in a carbenicillin-free environment (Fig. 1B, C). These results indicated that BGLUR133 and BGLUS35 strains had acquired resistance against carbenicillin.

I then assessed the minimum inhibitory concentration of carbenicillin to the respective TCS mutants. Both the BGLUR133 and BGLUS35 strains tolerated a high concentration of up to 150 μg/ml of carbenicillin (Fig. 2). These two mutants were resistant to other β-lactam antibiotics, penicillin-G, and ampicillin, but in contrast to the antibiotic-sensitive wild-type BGR1 (Fig. 3).

Genetic complementation of *gluS* and *gluR* (BGLUS35C and BGLUR133C, respectively) restored β-lactam sensitivity to the strains (Fig. 1A, B, C), thus affirming that the observed phenotypes were due to mutations in GluS-GluR TCS.

II. Cell viability of *B. glumae* strains amidst β-lactam antibiotics

Since β-lactam antibiotics form osmotically fragile filamentous cells prone to cell lysis; I hypothesized that *gluS* and *gluR* mutants might preserve normal cell division and form viable cells upon exposure to carbenicillin even though the *gluR* but not *gluS* mutant forms filamentous cells in a plain LB broth as shown in chapter 1. To test this hypothesis, the nature and viability of the wild type, the TCS mutants BGLUS53 and BGLUR133, alongside their respective complemented strains, BGLUS35C and BGLUR133C with carbenicillin treatment, were assessed. When a mixed stain assay of SYTO 9 green-fluorescent and red fluorescent propidium iodide nucleic acid was performed to highlight the viable and dead cells, respectively, after 12 hours of carbenicillin treatment, BGLUS35 and BGLUR133 formed heterogeneous cell cultures of both live (green) and dead (red) filamentous and normal rod-shaped cells in contrast to forming red fluorescing filamentous cells in the wild-type BGR1 (Fig. 4). Interestingly, after 24 hours of incubation, the mutant cultures were dominated by green fluorescing rod-shaped cells, while all filamentous cells appeared to die off (Fig. 4). The susceptible phenotype towards

carbenicillin was restored by the respective complemented mutant strains (Fig. 4), indicating that the altered GluS-GluR TCS triggered resistance to carbenicillin antibiotics in *B. glumae*.

III. Increased β-lactamase activity in GluS-GluR TCS mutants was responsible for the acquired resistance to carbenicillin

To determine the reason behind the observed resistance phenotype, I hypothesized that mutations in the GluS-GluR TCS could have triggered the production of β-lactam hydrolytic enzymes known as β-lactamases. When the activity of β-lactamases was assessed by observing the ability of the respective cell lysates to hydrolyze nitrocefin, a β-lactamase substrate, BGLUS35 and BGLUR133 cell lysates hydrolyzed nitrocefin from both carbenicillin-rich and plain LB cultures yielding a red color from yellow, a sign of complete hydrolysis (Fig. 5A). On the other hand, cell lysates of the wild type from plain LB depicted some degree of substrate hydrolysis, yielding an orange-like color. Since indistinguishable yellow color was observed in the buffer control, I concluded that the wild type lysate from carbenicillin-rich LB failed to hydrolyze nitrocefin (Fig. 5A).

Furthermore, I quantified the total β-lactamase produced by measuring the OD_{450} of the nitrocefin hydrolysates. As expected, higher OD_{450} readings were recorded for the BGLUS35 and BGLUR133 strains (Fig. 5B, C). Low readings

were recorded for the wild type strain, which decreased in the presence of carbenicillin near zero (Fig. 5B, C). Although β-lactamase expression in the BGLUS35 and BGLUR133 strains appeared to have reached saturation after 12 hours in plain LB (Fig. 5B), I observed a steady increase in β-lactamase expression over the time evaluated with carbenicillin treatment (Fig. 5C). The complemented BGLUS35C and BGLUR133C strains reinstated the inability of strains to hydrolyze nitrocefin (Fig. 5).

According to the genetic information, *B. glumae* BGR1 harbors four classes of β-lactamases, these include: β-lactamase class A protein (BGLU_2G14000), Metallo-β-lactamase superfamily protein (BGLU_2G09950, BGLU_1G16940, and BGLU_1G21360), Class C protein (BGLU_2G06860), and class D (BGLU_2G15400). It was, therefore, important to highlight the exact family involved in the increased β-lactamase activities. Inference came from quantitative PCR analysis that showed a significant increase of genes, BGLU_1G21360 and BGLU_1G16940, encoding MBLs in BGLUS35 and BGLUR133 compared to the wild type strain (Fig. 6A). The complemented strains, BGLUS35C and BGLUR133C, reverted MBL expressions to comparable levels to those in the wild type. The rest of the β-lactamase classes were not significantly affected by GluS-GluR mutations (Fig. 6A). I further evaluated whether GluR directly controls the expression of MBL genes by using EMSA with the putative promoter regions of

BGLU_1G21360 and purified His-tagged GluR (GluR-His) (Fig. 6B). The direct binding of GluR-His to the putative promoter regions of BGLU_1G21360 showed that GluR directly regulates the expression of MBLs (Fig. 6C).

IV. BGLUS35 and BGLUR133 possessed elevated expression of PBPs

In a susceptible scenario, β-lactams form irreversible covalent bonds with PBPs, arresting the cell wall assembly process. Therefore, I reasoned that PBP gene expression might be elevated in the *gluS* and *gluR* mutants to produce more PBPs. To determine how GluS-GluR TCSs influence the expression of genes encoding PBPs, I quantified PBP gene expression levels in the respective strains using qRT-PCR analysis. The results showed that expression levels of PBPs in the TCS mutants were significantly higher than that of the wild type (Fig. 7A). The complemented strains maintained low PBP levels comparable to the wild type level (Fig. 7A). When I assessed the ability of GluR-His to bind to the selected putative promoter regions of PBP genes with EMSA analysis, GluR-His bound to the putative promoter regions of BGLU_1G13280 and BGLU_1G04560, indicating the repressor role of GluR to the genes (Fig. 7B, C).

When fluorescent penicillin, Bocillin FL (Boc-FL), was used to visualize and quantify uninhibited PBPs after exposure to carbenicillin antibiotics, the BGLUS35 and BGLUR133 strains contained a significant number of uninhibited PBPs

preeminently shown by green fluorescence or by fluorescence intensity in comparison to the attenuated levels in the wild type strain (Fig. 8A, B). The complemented strains, BGLUS35C and BGLUR133C, gave responses similar to the wild type (Fig. 8A, B).

DISCUSSION

β-lactams interact with the bacterial cell wall assembly by covalently modifying the active sites of PBPs (Park and Strominger, 1957). This alteration inhibits the activities of the enzymes, damages the integrity of the cell wall, and leads to cell lysis (Park and Strominger, 1957; Cho *et al*., 2014). Despite the fact that β–lactams are among the most effective antibiotics, they are plagued with problems of resistance. Among various mechanisms to gain antibiotic resistance in bacteria, I found unexpected roles of TCSs in β-lactam antibiotic resistance in *B. glumae*, whereas TCSs in other bacteria have been known to play positive roles in acquiring antibiotic resistance (Arthur *et al*., 1992; McPhee *et al*., 2006; Hong *et al*., 2008; Gooderham and Hancock, 2009; Zamorano *et al*., 2014; Li *et al*., 2016). In contrast to the positive roles of TCSs in antibiotic resistance, the presence of GluS-GluR TCSs conferred sensitivity to β-lactam antibiotics in *B. glumae*. As such, it is known that mutations in *Staphylococcus aureus* WalK-WalR TCS induce resistance to multiple drugs of vancomycin and daptomycin (Howden *et al*., 2011). It would not be surprising to see more similar cases in roles of other TCSs in bacteria.

The consistently observed feature of the β-lactam antibiotic effect is the production of osmotically fragile filamentous cells prone to lysis and ultimately cell death (Kong *et al*., 2010). Analysis of the wild-type BGR1 at the onset of

carbenicillin treatment exhibited similar phenotypes. While the wild type was inhibited entirely, only the filamentous cells in the mutant strains were susceptible to the antibiotics leaving behind a homogeneous culture of normal and viable rod-shaped cells in *gluS* and *gluR* mutant cultures. Considering that the *gluS* mutant forms normal cells, unlike filamentous cells of the *gluR* mutant in LB without carbenicillin treatment (chapter 1), it was believed that the filamentous cell formation of both *gluS* and *gluR* mutants was due to carbenicillin treatment as observed in wild-type cells. This observation led to the hypothesis that mutations in GluS-GluR switched on the resistance mechanism towards β-lactams. The short-lived filamentous cells could have resulted from the acclimatization period as the strains rewired for an appropriate response.

A question that arises from this study was how mutations in the GluS-GluR TCS cluster impact the function of the regulator leading to β-lactam resistances. According to Wilke *et al*. (2005), β-lactam resistance is associated with the production or induction of β–lactamases, reinforcement of the target site in PBPs, and modification of transportation channels. This study discovered that mutations in the GluS-GluR cluster induced a nascent turnover β-lactamase that significantly improved hydrolytic activity in *B. glumae*. In the presence of carbenicillin, increasing β-lactamase production in the TCS mutants could suggest acclimatization and increased resistance to the antibiotics over time.

β-lactamases are grouped into four distinct classes dubbed A-D based on DNA sequences and catalytic requirements (Poole, 2004; King *et al*., 2017). Class A, C, and D are classified as metal-independent enzymes and utilize an active site serine for their hydrolytic activities (King *et al*., 2017). Class B enzymes, also known as MBLs, need zinc ions to synchronize a nucleophilic hydroxide that facilitates the opening of the β-lactam ring (King *et al*., 2017). Secretion and zinc ion-dependency of β-lactamase are important to understand β-lactam resistance in bacteria (King *et al*., 2017), and extracellular zinc ion-dependent MBLs degrades all groups of β-lactam antibiotics except monobactams (Walsh et al., 2005; Sacha *et al*., 2008). Among annotated MBLs in *B. glumae*, one MBL encoded by BGLU_1G21360 was found to be responsible for improved hydrolytic activity in the *gluS* and *gluR* mutants. However, it is not known whether the MBL encoded by BGLU_1G21360 is secreted and dependent upon zinc ion.

Previously, the induction of β-lactamases has been thought of as an indirect response to β-lactam-mediated cell wall damage (Hofer, 2016). But over time, bacteria have evolved and adopted less costly defense mechanisms such as using TCSs that sense antibiotics and direct an appropriate response without damaging the cell wall integrity. A good example is shown in the enteric pathogen, *Vibro parahaemolyticus*, where a conserved VbrK-VbrR TCS was reported to regulate the expression of β–lactamases in response to β-lactam exposure, and mutations in this TCS yielded β-lactam susceptible strains (Li *et al*., 2016). Despite this study

presenting a contrary scenario, I think that β-lactamases were simply produced more in the *gluR* and *gluS* mutants than wild-type BGR1 without damaging the integrity of the cell wall. My results demonstrate an example of how mutations in GluS-GluR TCS confer resistance against β-lactam antibiotics without damaging the cell wall integrity.

The inhibition of PBPs, which disrupts the equilibrium between peptidoglycan (PG) synthases and the action of PG hydrolases operating on expanding the cell matrix, is widely accepted as the mode of action of β-lactams (Tomasz, 1979). Expression of genes encoding PBPs was significantly higher in the β-lactam resistant BGLUS35 and BGLUR133 than in the wild type, which was not unexpected. The presence of many uninhibited PBPs after carbenicillin treatment further confirmed the β-lactam resistance phenotypes of the TCS mutants. This implied that exceeded expression of PBP allowed the mutants to overcome carbenicillin treatment and guaranteed continued cell division in the antibiotic environment. A similar mechanism as to how bacteria gain antibiotic resistance was known in *S. aeureus* (Wu *et al*., 2001; Sauvage *et al*., 2002; Wilke *et al*., 2005). This bacterium exerted resistance against β-lactam antibiotics through generating β-lactam insensitive PBPs (Wu *et al*., 2001) or increasing the level of PBP expressions (Sauvage *et al*., 2002; Wilke *et al*., 2005). My findings belonged to the latter case, and it would not be surprising to see more similar cases in other bacteria.

To support that GluR interacts with the key factors of β-lactam antibiotics resistance, I identified a conserved inverted repeat sequence in the upstream sequences of MBL (BGLU_1G21360) and PBPs (BGLU_1G13280) and BGLU_1G0456) (Fig.9). These discovered sequences were comparable to those observed in the upstream regions of *ftsA* and *ftsZ* in chapter 1.

While multiple cellular targets have previously been conveyed as an ideal strategy to minimize the incidence of mutational resistance (Silver, 2011), this study shows that bacteria also can devise various resistance mechanisms to counteract the drug effect. Therefore, my study undermines the benefits of multicellular targeted therapy previously discussed under the assumption that bacteria cannot make mutations that can trigger multiple resistance mechanisms. Thus, the principle learned in this study is that using a central system such as TCS as a therapeutic target can link stronger resistance in bacteria because it allows bacteria to hide behind possible therapeutic targets and store reserve drug resistance mechanisms. Although GluS-GluR mutations improve the fitness of *B. glumae* in a β-lactam environment, they also jeopardize the bacterial cell division processes as presented in chapter 1. Therefore, it is interesting to note the kind of evolutionary process this bacterium goes through after exposure to various environments since GluS-GluR TCS mutations cause various phenotypic changes affecting the fitness ability.

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Table 1. List of primers. (F- forward primer; R – reverse primer**)**

Primers	Sequence (5’ to 3’)
PBP1A-F	CTCGTAGTCCATCAGGCCG
PBP1A-R	CGCGCAATACAAGGACGAAA
PBP1-F	CGAGGCTATCGACGACGC
PBP1-R	CCACGAACTGCACCTCCAC
PBP2-F	ATCAGGAATACGGCATCGGC
PBP2-R	GCGACATCGACAGCCCATAG
PBP2A-F	ACATCACCTACGCCAACCRG
PBP2A-R	CGGGTAATTGACGAGCGAGA
bmlA-F	TTGTCGTATCCGGCGTCCT
bmlA-R	ATGCTCGGCAATACCACCG
MBL1 -F	GAGGACAGCCGGCCATTCT
MBL1-R	CTGATCCTGATCGAGCCCGTG
MBL2-F	GTGGTGCCGTCGAGTGTG
MBL2-R	TGGATCAACGACGAACCCAG
AmpC-F	GCCAGACATTTCGGCTCTTTC
AmpC-R	GTTCGAATCGGCACCTTTCC
1g21360p-F	GCGGCGTCGGGGCAGGCATC
1g21360p-R	GCTGCCGGTCGCGCTGCGTG
1g13280p-F	CCTTCTCGCTTCCGATGATA
1g13280p-R	TGGCGGGCGCTGCGGCGACA
1g04560-F	ATCAGCAGCGGCAGATCTTC
1g04560-R	CTCGACCCGAACTTGACGTT

Fig. 1. Mutations in GluS-GluR TCS induce β-lactam antibiotics resistance in *B. glumae*. (**A**) Bacteria strains of the wild type, *gluS* mutant (BGLUS35), *gluR* mutant (BGLUR133), complemented mutant strains (BGLUS35C and BGLUR133C) grown in LB with 50 μg/ml carbenicillin were serially diluted and spotted on LB Agar. The plates were incubated for 2 days at 37ºC and then photographed. (**B-C**) Cell population density of the indicated bacteria strains in (**B**) LB and (**C**) LB containing carbenicillin was quantified by colony forming unit (CFU) count, and results are expressed as logarithmic values of CFU/ml. The error bars represent the standard error (SE) ranges of the experiments performed in triplicate.

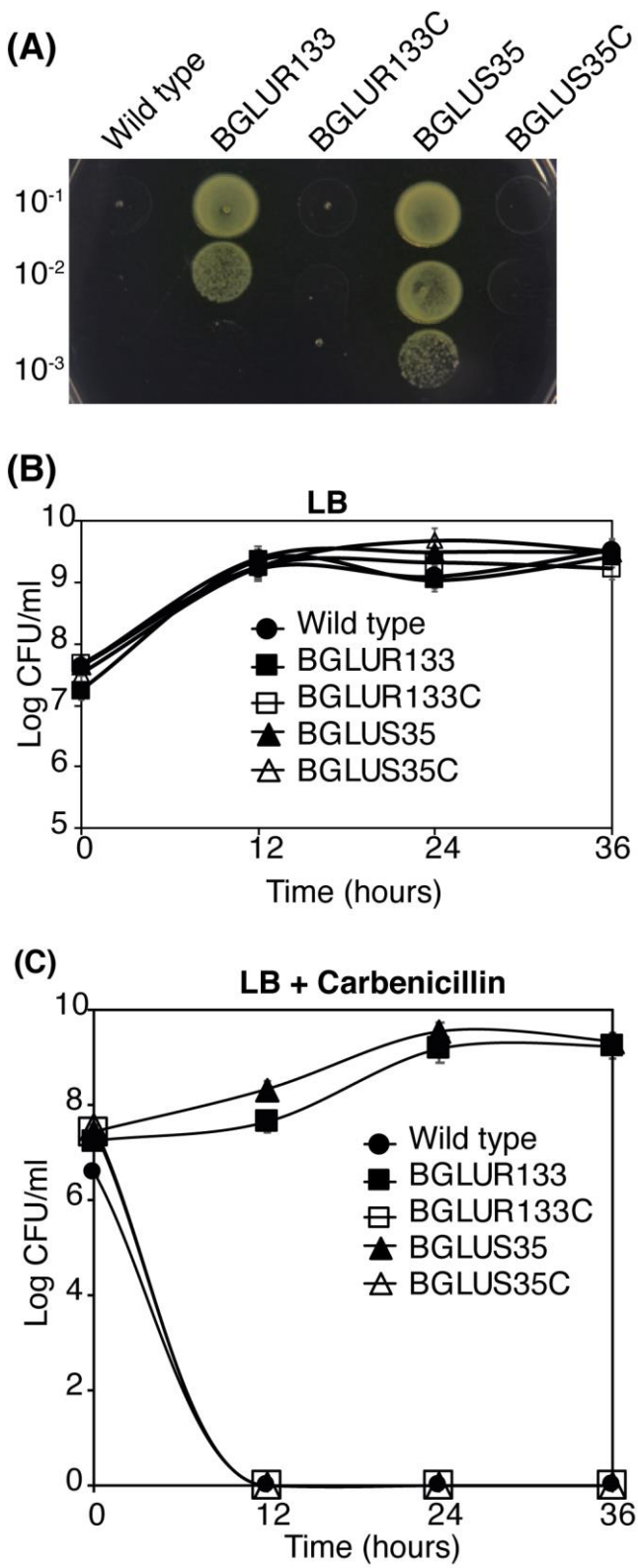
(A)
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C
10-1
10-2
10-3
(B)
LB
Log CFU/ml
10
9
8
7
6
5
0
12
24
36
Time (hours)
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C
(C)
LB + Carbenicillin
Log CFU/ml
10
8
6
4
2
0
0
12
24
36
Time (hours)
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C

Fig. 2. **Carbenicillin antibiotics tolerance in *B. glumae* strains.** GluS-GluR mutant strains can tolerate up to 150 µg/ml.

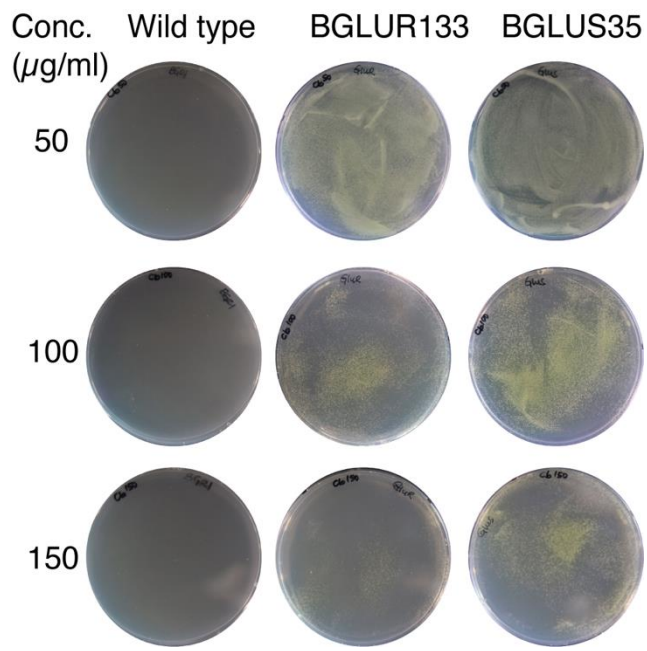

Fig. 3. β-lactam antibiotics resistance. The response of *B. glumae* strains to ampicillin and penicillin-G β-lactam antibiotics.

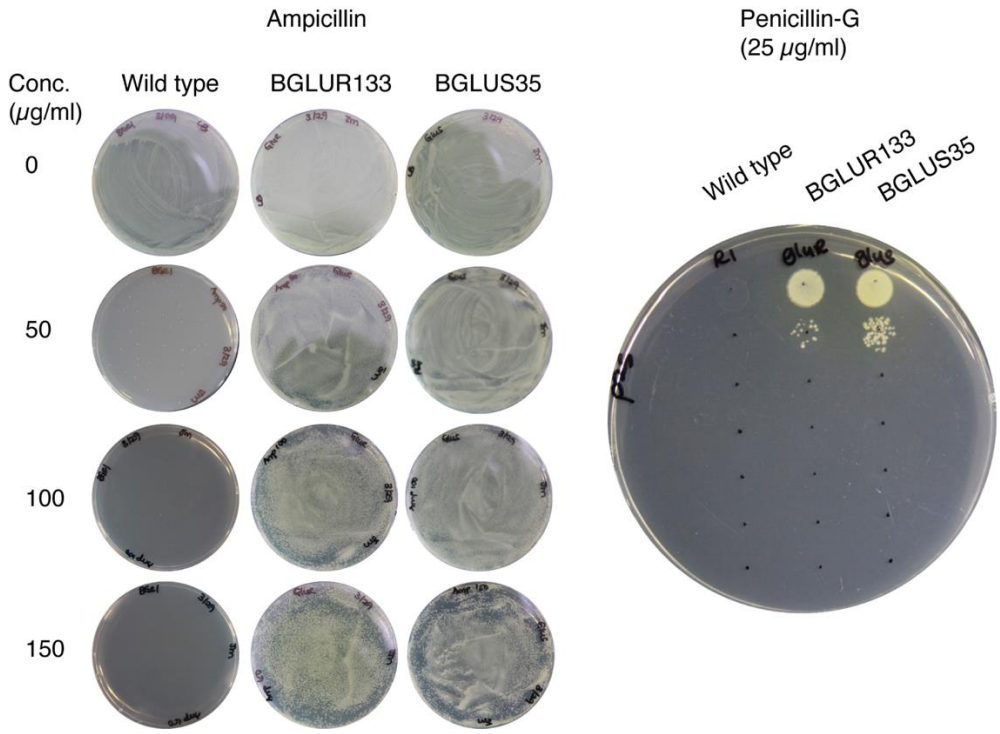

Fig. 4. The *gluS* and *gluR* mutants maintain viability in a carbenicillin environment. Cell viability of the wild type, TCS mutants (BGLUS35 and BGLUR133), alongside respective mutant complemented strains (BGLUS35C and BGLUR133C) cultured in LB with 50 μg/ml carbenicillin assessed by combination staining with propidium iodide (PI) and SYTO 9 green at the indicated time. Fluorescent images were obtained at excitation/emission wavelength 483/490-540 and 535/890-680 nm for green (live cells) and red (dead cells) fluorescence, respectively.

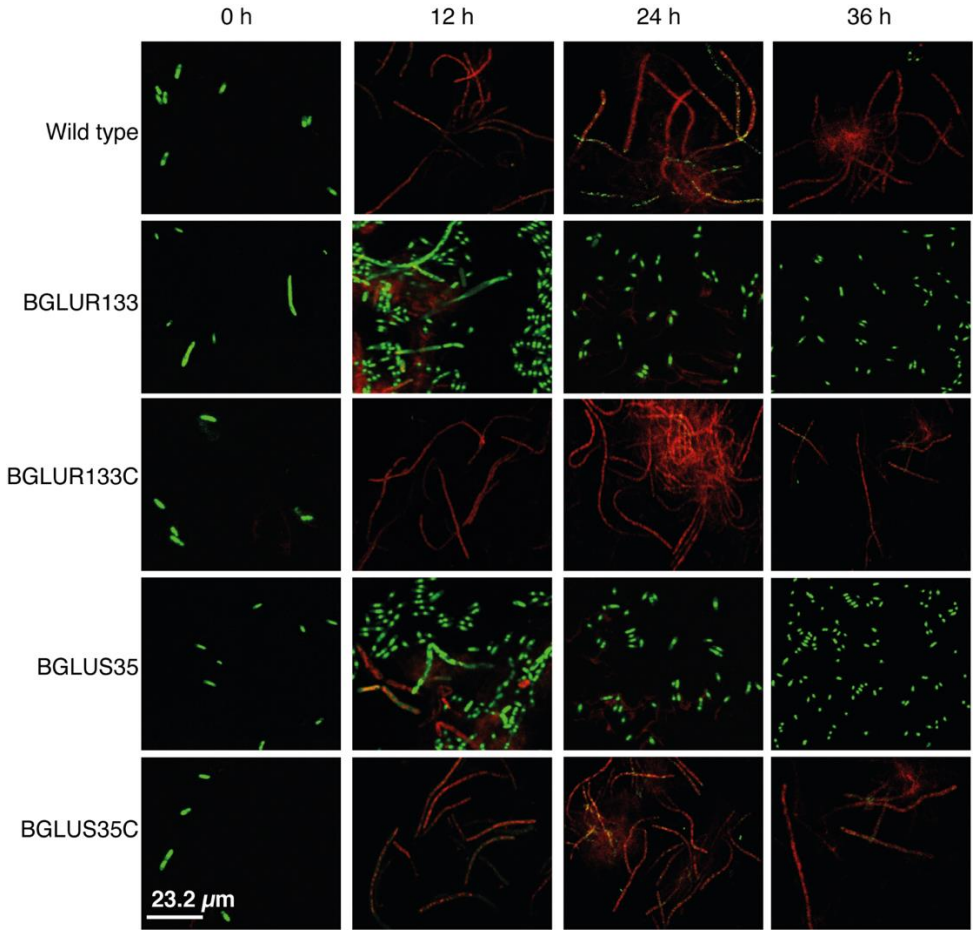
0 h
12 h
24 h
36 h
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C
23.2 μm

Fig. 5. The role of GluS-GluR in the expression of β-lactamases. (**A**) β-lactamase activity in LB and LB containing 50 μg/ml carbenicillin was measured by the hydrolysis ability of nitrocefin substrate, which changes color from yellow to red upon hydrolysis. (**B-C**) The relative amount of β-lactamase produced by each strain in (**B**) LB and (**C**) LB with carbenicillin quantified at OD_{450} at the indicated time. Represented values are the mean ± standard error (SE) of triplicates. PBS means phosphate buffered saline, pH 7.4.

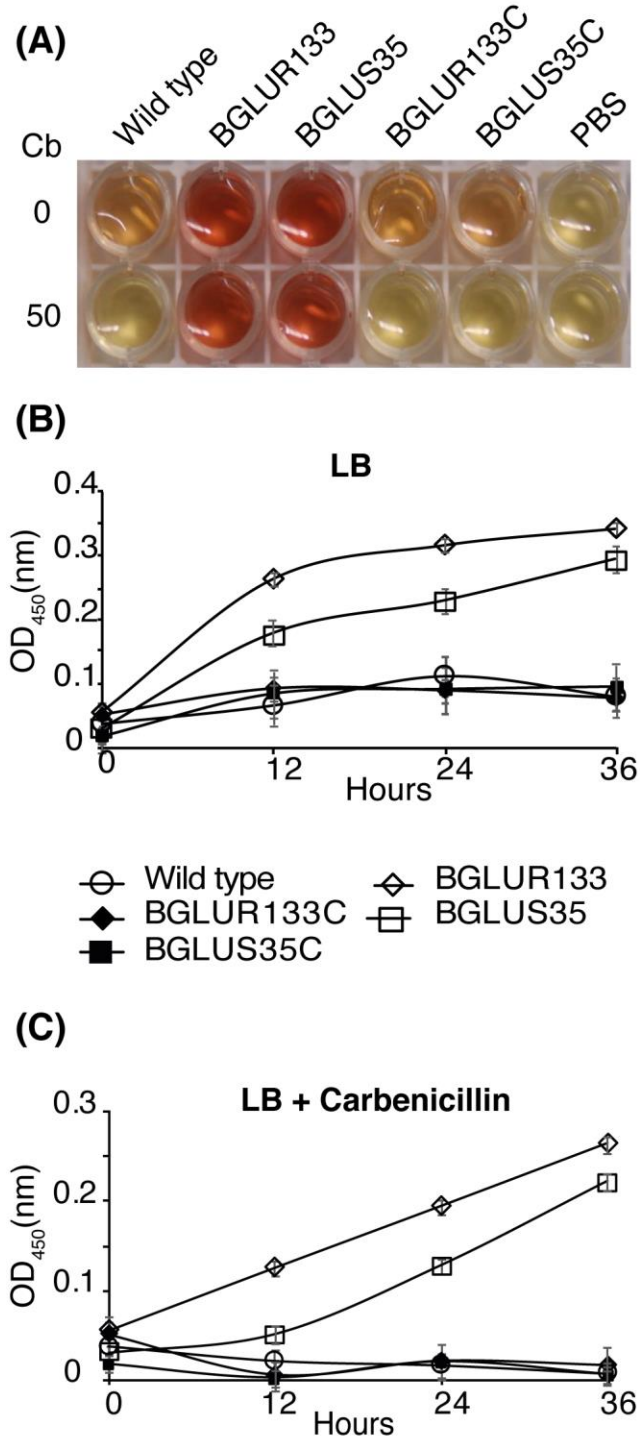
(A)
Wild type
BGLUR133
BGLUS35
BGLUR133C
BGLUS35C
PBS
Cb
0
50
(B)
LB
OD450(nm)
0.4
0.3
0.2
0.1
0
0
12
24
36
Hours
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C
(C)
LB + Carbenicillin
OD450(nm)
0.3
0.2
0.1
0
0
12
24
36
Hours

Fig. 6. The expression of β-lactamases in *B. glumae* BGR1 strains. **(A)** qRT-PCR analysis of the indicated β-lactamase genes was carried out on the wild-type BGR1, *gluS* mutant (BGLUS35), *gluR* mutant (BGLUR133), and the respective complemented mutant strains (BGLUS35C and BGLUR133C). Data represent the mean ± standard error (SE) of triplicates. Statistical analysis was performed using one-way ANOVA/Tukey's correlation for multiple comparisons. *, $p < 0.05$; ($F(19,40) = 3924.655$, $p = 0.00$). ns, not significant. (**B**) The gene map showing the putative promoter region of metallo-β-lactamase gene, BGLU_1G21360, for EMSA. (**C**) EMSA showing direct binding of GluR-His to the putative promoter region of BGLU_1G21360; 2 μM GluR-His, 2 nM labeled target DNA, 2 nM unlabeled *katE* non-competitor DNA, and 20 nM unlabeled target promoter DNA was used for EMSA.

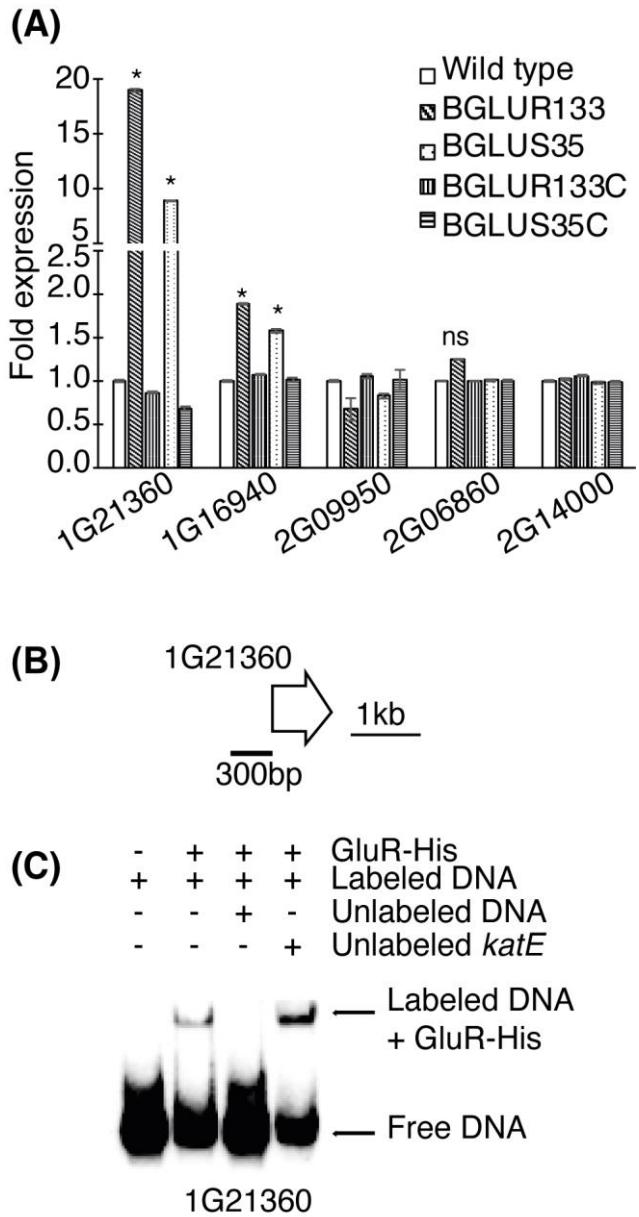
(A)
Wild type
BGLUR133
BGLUS35
BGLUR133C
BGLUS35C
Fold expression
20
15
10
5
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
ns
1G21360
1G16940
2G09950
2G06860
2G14000
(B)
1G21360
1kb
300bp
(C)
- + + + GluR-His
+ + + + Labeled DNA
- - + - Unlabeled DNA
- - - + Unlabeled katE
Labeled DNA
+ GluR-His
Free DNA
1G21360

Fig. 7. The role of GluS-GluR in the expression of penicillin-binding proteins (PBPs) in *B. glumae* strains. (**A**) qRT-PCR analysis of PBPs gene expression in the indicated strains. The gene IDs and names represented in the graph: BGLU_2G14710, Penicillin-binding protein; BGLU_1G02980, Penicillin-binding protein, 1A family; BGLU_1G04560, Penicillin-binding protein 2A family; BGLU_1G13280, Membrane carboxypeptidase (Penicillin-binding protein). Data represent the mean ± standard error (SE) of triplicates. Statistical analysis was performed using one-way ANOVA/Tukey's correlation for multiple comparisons. ***, $p < 0.0005$; **, $p < 0.005$; *, $p < 0.0005$; ($F(19,40) = 1550.875$, $p = 0.00$). (**B**) The gene maps showing the putative promoter regions of the selected genes for EMSA. (**C**) EMSA showing direct binding of GluR-His to the putative promoter region of BGLU_1G13280 and BGLU_1G04560; 1 μM GluR-His, 2 nM labeled target DNA, 2 nM unlabeled *katE* non-competitor DNA, and 30 nM unlabeled target promoter DNA were used for EMSA.

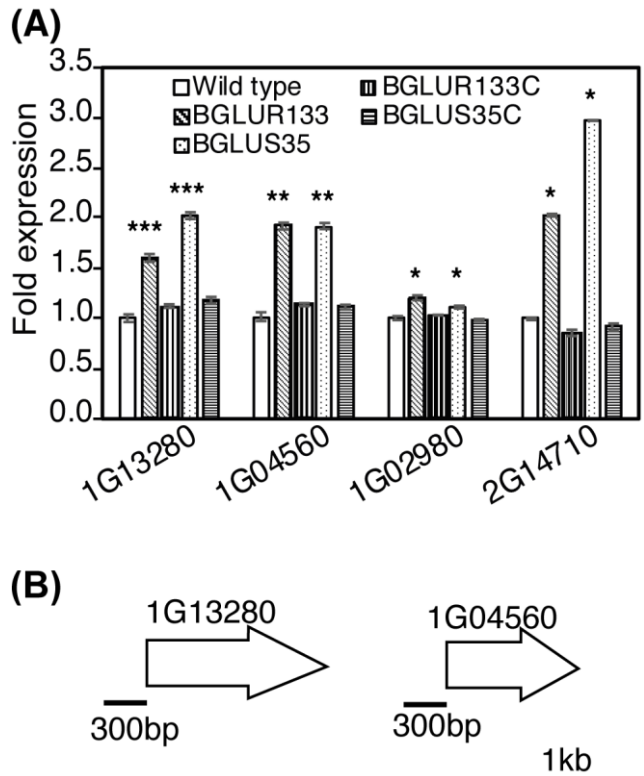
(A)
Wild type
BGLUR133
BGLUS35
BGLUR133C
BGLUS35C
Fold expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
**
**
*
*
*
*
1G13280
1G04560
1G02980
2G14710
(B)
1G13280
1G04560
300bp
300bp
1kb
(C)
- + + + - + + + GluR-His
+ + + + + + + + Labeled DNA
- - + - - - + - Unlabeled DNA
- - - + - - - + Unlabeled katE
Labeled DNA
+ GluR-His
Free DNA
1G13280
1G04560

Fig. 8. Characterization of penicillin-binding proteins (PBPs) in *B. glumae* strains. (**A**) Uninhibited PBPs in overnight LB with 50 μg/ml carbenicillin cultures were stained with Boc-FL and thereafter subjected to a cell membrane dye, FM-4-64. Green fluorescing spots depict uninhibited PBPs. (**B**) Quantification of PBPs as fluorescence intensity using Boc-FL. Fluorescent images and intensities were obtained at excitation/emission wavelengths 488/500-535 and 515/621-678 nm for Boc-FL and FM-6-64 dyes, respectively. Represented values are the mean ± standard error (SE) of triplicates. Statistical analysis was performed using one-way ANOVA/Tukey's correlation for multiple comparisons. **, $p < 0.05$; ($F(4,20) = 31.455, 31.506$, $p = 0.00$ for LB and LB with carbenicillin respectively).

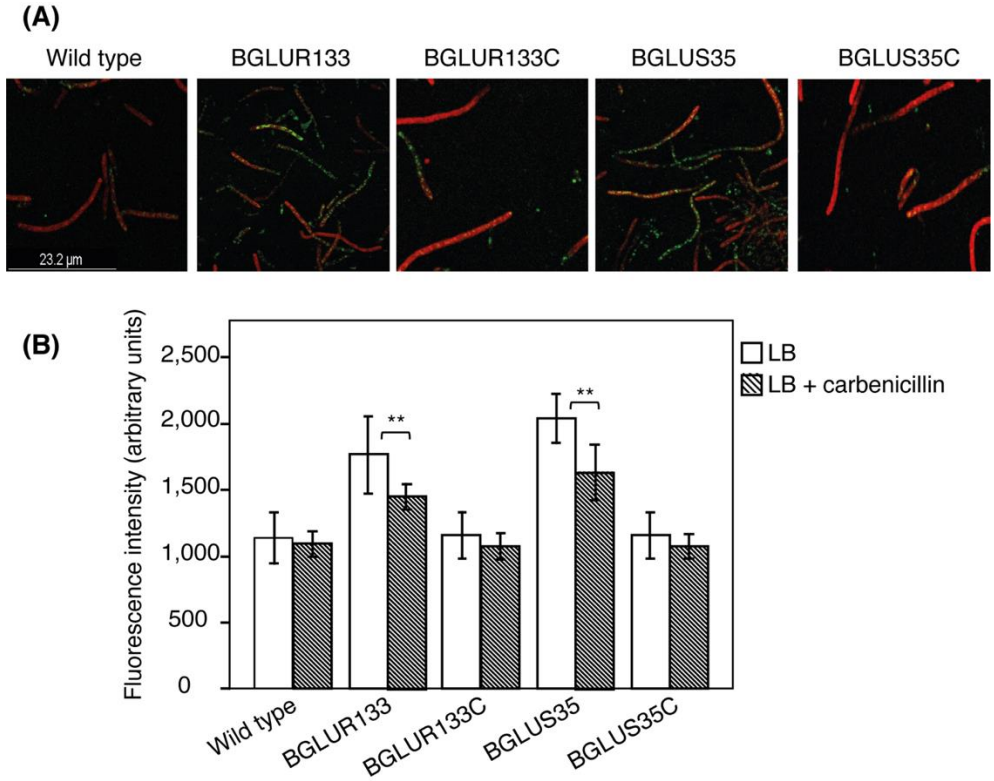
(A)
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C
23.2 µm
(B)
Fluorescence intensity (arbitrary units)
2,500
2,000
1,500
1,000
500
0
**
**
LB
LB + carbenicillin
Wild type
BGLUR133
BGLUR133C
BGLUS35
BGLUS35C

Fig. 9. Putative GluR binding site in MBL (BGLU_1G21360), PBP1 (BGLU_1G13280), and PBP2A (BGLU_1G04560) promoter regions. The conserved sequences present in upstream regions are highlighted. The inverted repeat sequences are indicated by the arrows.

CHAPTER 3

GluR and Lon Protease co-regulate Virulence and Fitness Functions of *Burkholderia glumae*

ABSTRACT

Two-component systems (TCSs) mediate the efficient adaptation of pathogenic bacteria to different environments, thus prerequisite for pathogenicity and survival. In chapter 1 and 2, the GluS-GluR TCS was identified as a regulator of cell division and β-lactam antibiotic resistance; cell division was disrupted in the absence of the response regulator GluR, but not its cognate sensor kinase GluS. In this chapter, I found that the *gluR* mutant did not cause hypersensitivity (HR) in non-host tobacco plants and exhibited attenuated disease symptoms in host rice plants, unlike the *gluS* mutant that maintained phenotypes similar to the wild type. Since HR is associated with type III secretion systems (T3SSs) and Lon protease negatively regulates T3SSs in Luria-Bertani (LB) medium, I created *lon* mutations to establish the relationship between GluR and T3SS. Mutation of *lon,* activated the T3SS coding genes of *hrpB, hrpG,* and *hrcC,* in the wild type but not in the *gluR* mutant in LB medium. Adding crude plant extract to LB medium to mimic apoplast conditions promoted *hrpB* and *hrpG* gene expression in the wild type, but not in the *gluR* mutant. Furthermore, I show that GluR, through a direct interaction with the gene encoding a manganese catalase, *katM,* causes resistance to hydrogen peroxide killing. Activation of *katM* catalase activities may boost full pathogenicity of *B. glumae*, as the *katM* mutant, like the *gluR* mutant, displayed reduced disease symptoms in rice plants. These findings suggest that GluR, as a regulator of T3SSs

and catalase activities, interacts with Lon protease to enable *B. glumae* adapt and survive in the host environment by regulating virulence and enhancing fitness to toxic reactive oxygen species.

INTRODUCTION

Two-component systems (TCSs) are common signal transduction devices in bacteria that allow them to generate adaptive responses to environmental stimuli via changes in gene expression. During the infection cycle, pathogenic bacteria encounter various microenvironments such as pH, nutrient availability, quorum sensing signals, or osmolarity, and their ability to efficiently adapt to different niches is often mediated by TCSs (Beier and Gross, 2006), which can thus be considered an essential prerequisite for their pathogenicity. The two components are a histidine sensor kinase that detects an external signal, and a response regulator which regulates a biological response by changing the expression of target genes (Beier and Gross, 2006). Although the involvement of TCSs in bacterial pathogenicity has been described in various species (Bronner *et al*., 2004; Uhl and Miller, 1996; Perraud *et al.*, 1998; Karki *et al.*, 2012), a thorough grasp of the regulation remains elusive. TCSs, like other signaling networks that regulate bacterial virulence, such quorum sensing, and epiphytic traits (Deng et al., 2009), are sometimes intricately liked to biological systems, such as the type III secretion system (T3SS) (Xiao *et al*., 2007; Deng *et al*., 2014; Deng *et al*., 2010; Chatterjee *et al*., 2003).

Through the remodeling of T3SSs, bacterial pathogens track the effective invasion of host tissues and adjust their metabolism, physiology, and expression of

virulence factors accordingly, for a successful invasion (Deslandes and Rivas, 2012). Galán and Waksman (2018) reported that the T3SS, which is encoded by a group *of hrp/hrc* genes, serves as a conduit for transmitting a variety of effector proteins into plant cells, resulting in the induction of either a hypersensitive response (HR) in resistant non-host plants or pathogenicity in susceptible plants. Examples of TCSs reported to improve the fitness of T3SSs include GacAS, RhpRS, and CvsSR of *Pseudomonas syringae* (Chatterjee *et al*., 2003; Xiao *et al*., 2007; Deng *et al*., 2014; Deng *et al*., 2010; Fishman *et al*., 2018).

Despite the identification of T3SS regulators, the inhibited expression of these genes in rich medium (Hutcheson *et al*., 2001; Jovanovic *et al*., 2011) was still baffling. The discovery of an ATP-dependent Lon protease that degrades and/or suppresses T3SS genes and their activators (Bretz *et al*., 2002) was a breakthrough. Depending on the bacterial species, the proteolytic effect of Lon will control T3SS either negatively (Takaya *et al*., 2005) or positively (Jackson *et al*., 2004). In addition to directly targeting T3SS genes (Bretz *et al*., 2002), Lon protease has been shown to interact with T3SS regulatory factors such as TCSs (Zhou *et al*., 2016). Examples of Lon regulated TCS-T3SS pathways include *rhpS* of *Pseudomonas syringae* (Zhou *et al*., 2016), *cpxR* of *Salmonella enterica* (De la Cruz *et al*., 2015), and *cpxA* of Enterohemorrhagic *Escherichia coli* (EHEC) (De la Cruz *et al*., 2016).

Even though significant progress has been made in understanding T3SS, many economically important pathogens such as *Burkholderia glumae* remain

understudied. *B. glumae* is the etiological agent of rice panicle blight causing up to 75% yield losses when weather conditions are favorable (Zhou-qi *et al*., 2016). This bacterium is an example of complex pathogens that uses a variety of factors to maintain pathogenicity, including toxoflavin, quorum sensing (QS), pellicles, oxalate, and motility, which our lab has previously investigated (Kim *et al*., 2004; Kim *et al*., 2007; Kwak *et al*., 2020; Goo *et al*., 2012). In this chapter, I add to the increasing body of knowledge about *B. glumae* virulence mechanisms. I investigated the relationship between *B. glumae's* TCS, Lon protease, and T3SS in this study. Hitherto, our lab discovered that *B. glumae* strain BGR1 has a T3SS that is essential in pathogenicity (Kang *et al*., 2008), but little is known about how it works. In this chapter, not only do I show that GluR regulates virulence and bacterial fitness independently of GluS, but I also show that GluR and Lon protease have a molecular and functional interaction in controlling the T3SS genes in *B. glumae* BGR1.

MATERIAL AND METHODS

I. Bacterial strains and growth conditions

The bacterial strains, plasmids, antibiotics, and the growth conditions used in this chapter are described in chapter 1 (Materials and Methods section I). Additional strains are listed in Table 1 of this chapter.

II. DNA manipulation, sequencing, and mutagenesis

Basic DNA manipulations were done as described in chapter 1 (Material and Methods section II). The TCS mutants (BGLUS35 and BGLUR133) and the *gluR* mutant complemented strain, BLGUR133C used in this chapter were generated in chapter 1 (Method and Methods section III). The *lon* mutant (BLONN) and *lon* mutant complementation, BLONC, were previously generated in our lab. The *lon/gluR* double mutant (BGLULON) was constructed by introducing pBGH13 clone carrying Tn*3-gusA* insertions in the *gluR* gene into BLONN via tri-parental mating (Figurski & Helinski, 1979).

To construct *katM* mutant strain, BKATM, I used pLAFR3 derivatives of pBGM1 cosmid clone carrying *katM* gene (BLU_2p0200), which was mutagenized using Tn*3-gusA* as described in chapter 1 (Method and Methods section III). The *katM* mutant complementation, BKATMC, was generated by digesting pBGM1 with *Bam*H1 and *Hind*III followed by ligation into pLAFR3 to form pBGM2. The

pBGM2 plasmid was introduced into BKATM via tri-parental mating (Figurski & Helinski, 1979) to produce BKATMC.

III. HR elicitation, virulence assay, and bacterial population

The bacteria cells (10^8 cells/ml) suspended in sterile distilled water were inoculated into four weeks old tobacco leaves for hypersensitive response (HR) (Klement, 1963) and Milyang-23 rice plants to assess their disease effect. The plants were kept in the growth chamber HB-303D-L (Hanbaek Scientific, Korea) for 24 hours (HR test) and 12 days (virulence test) at 30^{o}C and 16/8-hour day/night respectively. Disease symptoms in rice were quantified by ImageJ 1.53a software (USA) by measuring pixels of the diseased area using the wild type as a standard and scored using a scale: 0 = healthy plant and 1= full symptoms from the wild type. Disease index was calculated (disease index = disease pixels/disease pixels from the wild type).

For the bacteria population assay, at a three-day interval for 12 days, the infected rice stems were ground in 1 ml distilled water and serial dilutions plated on LB agar medium. The bacteria population was quantified using the direct colony count (CFU) method and population density expressed as the logarithm of CFU/ stem.

IV. Toxoflavin assay

Toxoflavins from the wild-type BGR1 and the TCS mutants were extracted from overnight cell cultures using chloroform following previously described methods (Yoneda *et al.*, 1971). Chloroform extracts were then dissolved in dimethyl sulfoxide (DMSO) and analytical TLC carried out on a silica gel 60 TLC plate (Merk, Germany) using chloroform/methanol (95:5, v/v) and thereafter visualized under UV light at 365 nm.

V. Autoinducer assay

The QS signal production assay was performed following previously described procedures (Kim *et al*., 2004), with a few modifications. The HSLs were extracted from overnight bacteria cultures by mixing the cell-free supernatants and ethyl acetate (1:1). The ethyl acetate extracts were evaporated using a rotary evaporator below 40^{o}C and the residues reconstituted in 10 µl of DMSO. A 5 µl sample was then dropped on LB agar medium containing *Chromobacterium violaceum* biosensor and the plates incubated at 28^{o}C overnight.

VI. Preparation of plant extracts

Tobacco leaves that were four weeks old were used to make crude plant extract. The leaves were washed under running water, dried at 37^{o}C, and dry weight

determined before being crushed into powder. The powder was soaked in 95% methanol for 48 hours at room temperature in a 6:1 ratio, Methanol: plant powder, respectively. The plant residues were filtered out, and methanol was removed using rotor evaporation, resulting in a vicious mass that was suspended in water and filter sterilized. To mimic apoplast conditions in LB medium, I used 100 g/ml crude extract.

VII. RNA extraction and qRT-PCR

Total RNA was isolated from *B. glumae* strains and cDNA was synthesized as described in chapter 1(Material and Method section VI). Using primers listed in Table 2, designed to amplify the specific genes, transcription levels were determined as described in chapter 1.

VIII. Hydrogen peroxide sensitivity assay

A cell volume of 4×10^8 cell/ml from overnight LB cultures was sub-cultured into fresh LB containing 0, 0.5, 1, 1.5, 2, 2.5, and 3 mM H_2O_2 and grown for 24 hours. Using the CFU count method, the bacterial growth was quantified.

IX. Catalase activity assay

Following previously established methods (Jittawuttipoka *et al*., 2009; Yu *et al*., 2016), the catalase activity assay was performed with a few modifications. Bacterial cells of 4×10^8 cell/ml from overnight LB cultures were sub-cultured into fresh LB containing 0 and 2 mM H_2O_2 and grown for 24 hours. The cells were chilled at 4^oC for 20 minutes and then collected at 6,000 g, resuspended in 50 mM potassium phosphate buffer (pH 7), and thereafter sonicated using SONICS vibra Cell (Sonics & Materials INC. Newtown, CT, USA). The cell extracts were separated by centrifugation at 12,000 g for 30 minutes and the upper layer which contained the proteins was obtained. Using a 10 mM H_2O_2, the optical density of the mixture was measured at 240 nm. The catalase activity was calculated using an extinction coefficient of 43.6 M/cm at 240 nm. One unit of catalase activity was defined as the catalase activity to required decompose H_2O_2 under the assay conditions.

X. Electrophoretic mobility shift assay (EMSA)

The EMSA assay was performed using histidine-tagged Lon (Lon-His), HrpB-His (both previously prepared by our lab) and GluR-His that was prepared in chapter 1.

Using primer sets hrpBp-F/R, katMp-F/R, and glurp-F/R listed in Table 2, putative promoter regions of the respective genes were amplified and labeled with

biotin as described in chapter 1 (Methods and Material section XII). The respective competition assays were carried out as described in chapter 1 (Material and methods section XII).

XI. Protein *in-vitro* degradation assay

The degradation assay was performed as previously described (Zhou *et al*., 2018) with a few modifications. 5 µg of His-tagged substrate (GluR-His, HrpB-His) were mixed with varying concentrations of His-tagged Lon protease (0, 5,10, 20 µM), 10 mM ATP in Lon degradation buffer (100 mM NaCl, 10 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0)). The reactions were incubated at 37°C for 1h. 5 µM purified α-casein was used as the control substrate. The reaction was stopped by adding SDS loading dye and denatured. The samples were then separated 10% SDS-PAGE and visualized using Coomassie blue G-250 stain.

XII. Statistical analysis

Statistical analysis was performed as described in chapter 1 (Material and Methods, section XIII). A p-value < 0.05 to indicate statistical significance was used.

RESULTS

I. Impact of GluS-GluR mutations on the virulence of *B. glumae*

Chapter 1 showed that the response regulator GluR, but not its cognate sensor kinase GluS, was needed for cell division. Since sets of genes could be linked to the cell cycle and virulence, I reasoned that GluR and/or GluS may be involved in the virulence process of *B. glumae*. To test this theory, I inoculated non-host tobacco leaves with the *gluS* mutant (BGLUS35), *gluR* mutant (BGLUR133), alongside the *gluR* mutant complementation, BGLUR133C, and the wild-type BGR1 to see if they could induce hypersensitive response (HR). HR was produced by BGLUS35 and BGR1, but not *gluR* mutant BGLUR133 (Fig. 1A). Furthermore, I injected the bacteria strains into host rice stems and observed for disease symptoms. As illustrated in Fig. 1B, BGLUR133 showed significant disease reduction with an index of 0.34 ± 0.15 compared to the severely diseased stems of BGR1 with a disease index of 1.0 ± 0.52. The complementation of the *gluR* mutant (BGLUR133C) restored the HR and disease phenotypes to those observed in the wild type (Fig. 1A, B). The disease index of BGLUS35 (0.73 ± 0.04) was, on the other hand, comparable to that of BGR1 (Fig. 1B), confirming the findings in chapter 1 that these two polycistronic genes are not a perfect functional pair.

The bacterial numbers of the wild-type BGR1 and the TCS mutants were similar and did not change significantly after inoculation into the rice plants for the 12 days

monitored (Fig. 1C), indicating that the growth and survival of the bacteria was not affected by the mutations in the GluS-GluR TCS cluster.

Next, I sought after the essential components for the GluR-meditated virulence. Using wild-type BGR1 as a control, I assessed the ability of the TCS mutants to produce toxoflavin and quorum-sensing (QS) signals. BGLUR133 and BGLUS35 both produced toxoflavins and QS signals comparable to BGR1 (Fig. 2), raising the question of how GluR regulates virulence in *B. glumae* BGR1.

To assess whether GluR functions of cell division and virulence were connected, I tested the HR of BGLUR133(pFtsA) and BGR1(pFtsA) (generated in chapter 1 with modified cell division functions). The BGR1(pFtsA) strain with abnormal cell division was able to induce HR in tobacco leaves, wheras the BGLUR133(pFtsA) strain with normal cell division was unable to do so (Fig. 3). These findings suggested that GluR's cell division and virulence functions were regulated independently.

II. GluR and Lon protease inversely regulate T3SS in *B. glumae* BGR1

The implied role of GluR in the pathogenicity of *B. glumae* raises the question of whether this response regulator is functionally complementary, separate, or overlapping with a type III secretion system (T3SS) inhibitor, Lon protease, which may be reflected by differences or similarities in the expression of T3SS genes. So far, my findings propose a duo function between GluR and Lon Protease in the

induction of T3SS. To test this hypothesis, I constructed a *gluR/lon* double mutant by introducing the pBGH13 clone carrying Tn*3-gusA* insertions in the *gluR* gene into the chromosome of BLONN and compared the HR phenotypes to the single mutants and the wild type. The BLONN and BGLULON strains, like BGLUR133 did not induce HR (Fig. 4A). BLONN mutant complementation (BLONC) restored HR just like the wild-type BGR1 did, however, complementation of the respective genes in the *gluR/lon* double mutant (BGLULON) had no effect on the strain's negative HR phenotype (Fig. 4A).

Using 16S *rRNA* gene as a control, I then investigated the bacteria strains' functions in the expression of selected T3SS genes of *hrpB, hrpG,* and *hrcC,* in LB medium. Since T3SS genes are only expressed in plant or apoplast-like conditions, no expression of the selected genes was expected in the wild-type BGR1 cultured in Luria-Bertani (LB) medium; a similar phenotype was observed in the BGLUR133 (Fig. 4B). It was also predictable that mutations in *lon*, a well-known T3SS suppressor, resulted in T3SS gene activation (Fig. 4B). Interestingly, BGLUR133 with a null *lon* gene (BGLULON) was unable to express *hrpB, hrcC*, and *hrpG* genes (Fig. 4B). By using the complementation gene strategy, the T3SS gene expression in BGLULON was recovered to that of *lon* mutant (BLONN) when the cosmid clone pBGH13 was present *in trans* in the *gluR* mutant (Fig. 4B). Complementation of the *lon* mutant in BGLULON strain did not affect the

expression of T3SS genes (Fig. 4B) posing a question of how Lon protease may be linked to the GluR function.

I also compared the response of the *lon* mutant (BLONN) in the host rice plants to the wild type. In comparison to the wild type, rice stems infected with BLONN showed minimal stem discoloration **(**Fig. 5A**)**. Quantification of bacterial populations in rice plants revealed that BLONN multiplied substantially slower than the wild type **(**Fig. 5B**)**, which could explain the reduced of disease symptoms **(**Fig. 5A**)**. The mutant complemented strain (BLONC) restored the wild type-like response (Fig. 5A, B**).**

III. Mutations of *gluR* halts T3SS gene induction in *in-vivo*

Since T3SS genes are expressed under plant apoplast conditions, to broaden the understanding of the GluR functional role, I simulated an *in-vivo* environment by growing the bacteria strains in LB medium supplemented with a crude plant extract and tested for *hrpB* and *hrpG* gene induction. Indeed an *in-vivo* like environment induced *hrpB* and *hrpG* genes in BGR1, unlike BGLUR133 which showed no expression of the T3SS genes (Fig. 6A). Reversibility of *gluR* mutations in BGLUR133C reinstated the strains' ability to induce the *hrpB* and *hrpG* genes in LB medium supplemented with plant extract (Fig. 6A).

To further demonstrate that T3SS gene expression is controlled by GluR, I performed EMSA on the putative promoter region of *hrpB* (Fig. 6B) and purified

His-tagged GluR (GluR-His). The binding of GluR-His to the putative promoter region of *hrpB* confirmed that GluR directly activates the expression of *hrpB* in *B. glumae* (Fig. 6C).

IV. Lon protease does not degrade but activates *gluR* and inhibits *hrpB*

Next, I assessed the interaction between Lon protease and the T3SS regulators GluR and HrpB. T3SS regulators are among the many Lon protease substrates that have been discovered so far. Although the connection between *gluR* and Lon protease was not yet clear, the ability of the *lon* mutant to express the natively inert T3SS genes in *B. glumae* in LB medium (Fig. 4B**)** led me to hypothesize that this protease acts similarly to the established roles in other bacteria species. Therefore, I tested Lon's ability to digest GluR and HrpB in an *in-vitro* environment containing purified forms of the proteins. To confirm the activity of Lon protease, I used purified α-casein as a control substrate. Lon did not degrade His-tagged GluR or HrpB, as shown in Fig. 7A. Lon Protease was present in an active form, as evidenced by the efficient degradation of α-casein (Fig. 7A). Interestingly, quantification of *gluR* gene expression in the *lon* mutant (BLONN) showed an over 5-fold reduction in the expression of *gluR* compared to the wild-type BGR1 (Fig. 7B). The complementation of the *lon* mutant significantly increased the *gluR* gene expression level (Fig. 7B), suggesting that Lon protease acts as an activator of GluR.

Next, I performed EMSA with the putative promoter regions of *gluR, hrpB* and purified His-tagged Lon (Lon-His) to further analyze the relationship between Lon with GluR and HrpB. The Fig. 7C shows the direct binding of Lon-His to the putative promoter regions of *gluR* and *hrpB* confirming a direct interaction of Lon protease with the genes.

V. GluR mediates resistance to H_2O_2 killing in *B. glumae*

TCSs enable bacteria to counter the toxic host environment and since GluR has shown to be important in the virulence of *B. glumae* (Fig. 1), I reasoned that this response regulator might be essential in mitigating around the host defense system that involves the production of toxic reactive oxygen species (ROS). To test this theory, I compared the growth ability of the TCS mutants (BGLUS35 and BGLUR133) with the wild type and the respective mutant complementation strain in increasing concentrations of H_2O_2. Contrary to the wild type, BGLUR133 was more sensitive to H_2O_2 (Fig. 8A). With the increasing concentration of H_2O_2, the cell numbers of the BGLUR133 were dramatically decreased until total inhibition was achieved at 3 mM H_2O_2 (Fig. 8A). The complementation of the *gluR* mutant (BGLUR133C) restored the wild type-like response (Fig. 8A). On the other hand, growth, and survival of BGLUS35 was not affected by the increasing H_2O_2 concentrations (Fig. 8A) indicating that GluR, and not GluS is essential in *B. glumae*'s viability under H_2O_2 stress

To further unveil the function of *gluR* in H_2O_2 degradation, I comparatively measured the catalase activity of BGR1, BGLUR133, and BGLUR133C when grown in LB medium with and without H_2O_2. The catalase activity of the BGLUR133 was significantly decreased compared to the wild type but was restored in BGLUR133C (Fig. 8B). Collectively, these findings suggested that GluR contributes to the catalase activities for H_2O_2 detoxification in *B. glumae*.

VI. GluR directly activates the activities of a manganese catalase, *katM*

Because GluR has been found to be essential in the catalase activities (Fig. 8), I investigated its interaction with *B. glumae*'s catalase genes. According to the genetic information of *B. glumae* BGR1, there are seven catalase genes: BGLU_2G03420, BGLU_2G19650, BGLU_2G21370, BGLU_2P0200, BGLU_1G00120, BGLU_1G14950, and BGLU_1G22650 dubbed *katM-2*, *katQ*, *katB*, *katM*, *katS*, *katQ*, and *katE*, respectively. To determine which catalase was under the control of GluR, I carried out qRT-PCR analysis of the catalase genes in BGR1, BGLUR133, and BGLUR133C. The assay showed a significant decrease in the expression of *katM* (BGLU_2P0200) in BGLUR133 compared to the wild type (Fig. 9A). The mutant complemented BGLUR133C on the other hand expressed *katM* to levels comparable to that in the wild type (Fig. 9A). And while there was no significant difference in the expression levels of *katB*, *katS*, *katQ*, and *katG*, *katE* on the other hand was highly expressed in the BGLUR133 (Fig. 9A).

To confirm that *katM* expression is directly controlled by GluR, I performed EMSA with GluR-His and the putative promoter region of *katM* (Fig. 9B). As shown in Fig. 9C**,** GluR-His bound to the upstream region of *katM*, suggesting GluR directly regulates *katM.*

VII. *katM* mutant is sensitive to exogenous H_2O_2

To confirm that *katM* was indeed responsible for the observed GluR-mediated H_2O_2 resistance, I constructed *katM* mutant strain by Tn*3-gusA* mutagenesis of pBGM1 cosmid carrying *katM* gene (Fig. 10A). This *katM* gene of *B. glumae* belongd to the manganese catalase family, encodes a 15 kDa protein that exhibited 93.3% similarities with another manganese catalase gene of *Burkhodelia gladioli* (Accession number WP_186098774.1). The pBGM1 clone carrying Tn*3-gusA21* insertion was marker exchanged into *B. glumae* BGR1 resulting in BKATM (*katM*::Tn*3-gusA*).

I then compared the catalase activity of BKATM with that in BGLUR133 and the wild-type BGR1. BKATM and BGLUR133 recorded significantly decreased catalase activities than BGR1 (Fig. 10B). Although there was no significant difference between the catalase activity of BKATM and BGLUR133 in plain LB medium, in the presence of 2 mM H_2O_2, the catalase activity in BKATM was reduced to levels significantly lower than those in BGLUR133 (Fig. 10B). Catalase activity in BKATM was restored to levels comparable to the wild type when the

plasmid pBGM2 carrying the *katM* gene was mobilized into the mutant in *trans* (Fig. 10B) thus confirming the observed phenotype was indeed a result of mutations in the *katM* gene.

VIII. *katM* mutant showed attenuated virulence

Previously, our lab discovered that one catalase gene, *katG* controlled by QS, was involved in the virulence of *B. glumae*. Similarly, I compared the virulence of BKATM to that of BGLUR133 and BGR1, as well as their respective mutant complementation. Both BKATM and BGLUR133 showed attenuated disease symptoms, with disease index of 0.1±0.01 and 0.09±0.01, respectively, which were significantly lower than the 1±0.15 disease index shown in wild-type BGR1 (Fig. 11). The mutant complemented strains re-instated the strains' disease severity comparable to BGR1 (Fig. 11).

DISCUSSION

The TCS has been linked to the virulence of many bacteria pathogens in an increasing number of studies (Bronner *et al*., 2004; Karki *et al*., 2012). In this chapter, I describe the role of the GluR response regulator in *B. glumae's* virulence mechanism and fitness to its host environment. In the absence of GluR, HR was impaired, disease symptoms were reduced, and T3SS genes (*hrpB, hrpG, and hrcC*) were not expressed. The bacterium's virulence was unaffected by mutations in the cognate sensor kinase, GluS. This observation was not surprising given that I discussed the unpaired activities of GluS-GluR in the process of cell division in chapter 1, and similar studies in other bacterial species have also been published (Stenson *et al.*, 2005; Nakayama *et al.*, 2003).

The virulence properties of *B. glumae* have been attributed to the production of a yellow pigment virulence factor – toxoflavins under the control of QS (Kim *et al*., 2004). I found that the TCS mutants and the wild type produced comparable amounts of toxoflavins and QS signals, indicating that GluR is not a component this system, thus raising the question of how GluR was involved in the virulence of *B. glumae*.

Like most bacterial pathogens, *B. glumea's* T3SS is induced in nutrient-poor conditions or the host environment (Rahme *et al.*, 1992), but inhibited in nutrient-rich conditions (Hutcheson *et al*., 2001; Jovanovic *et al*., 2011), and studies have

linked this to the presence of Lon protease (Bretz *et al*., 2002). Therefore, in this study, I used the wild type and *gluR* mutant strains with a null *lon* gene to investigate any link between GluR and the T3SS of *B. glumae*. Albeit the loss of HR and reduced disease symptoms, mutations of *lon* in the wild type activated the expression of the AraC-type transcriptional activator *hrpB*, as well as downstream genes of *hrpG* and *hrcC*, in the rich LB medium. However, similar to the *gluR* mutant (BGLUR133), *gluR/lon* double mutation (BGLULON) failed to induce the expression of these T3SS genes. We assume that the attenuated virulence of the *lon* mutant (BLONN) was related to its slow growth, similar findings were reported in *Agrobacterium tumefacien* (Su et al., 2006). Addition of crude plant extract to LB medium to imitate apoplast-like environment promoted the expression of T3SS genes in the wild type but not in the *gluR* mutant, suggesting that a functional GluR is required for T3SS induction. The direct binding of GluR to the putative promoter region of *hrpB* confirmed the GluR activator role of the T3SS. Although my findings are consistent with the previously reported RhpR-RhpS TCS of *P. syringae* that activates T3SS (Deng et al., 2014; Fishman et al., 2018; Xie et al., 2019), this study also presents Lon protease as an activator of GluR in *B. glumae*, in contrast to Lon's previously reported suppressor role of the RhpR-RhpS (Zhou et al., 2016). *P. syringae*'s *lon/rhpS* double mutant recovered the HR phenotype of *rhpS* (Zhou et al., 2016), however mutation of *lon* in *B. glumae*'s *gluR* mutant did not.

Purified Lon protease has been reported to control the T3SS by degrading T3SS activators (Bretz *et al*., 2002). Lon-His successfully bound to putative promoter regions of *gluR* and *hrpB* in this study, playing opposing roles as a *gluR* activator and a *hrpB* repressor. In comparison to other bacterial species such as *P. syringae* (Bretz *et al.,* 2002), *Salmonella enterica* serovar Typhimurium (Takaya *et al*., 2005), *Yersinia pestis* (Jackson *et al*., 2004), and *Xanthomonas citri* (Zhou *et al.*, 2018), Lon negatively regulates T3SS and degrades the activators. GluR and HrpB were unlikely substates of Lon protease because Lon protease did not degrade the respective proteins *in vitro*, implying that Lon proteolysis activities might not be needed for T3SS induction in *B. glumae*. To the best of our knowledge, this is the first report on Lon protease as a transcription regulator of its target genes.

Pathogenic bacteria also rely on their ability to counteract oxidative stresses such as H_2O_2 in order to survive and infect plant tissues (Cabiscol *et al.*, 2000). *B. glumae* is constantly exposed to H_2O_2 produced by toxoflavin autorecycling oxidation (Chun *et al.*, 2009), aerobic respiration, and the oxidative burst of the host plant cells during plant-pathogen interaction, making their catalases essential for protection against oxidative-stress damage (Yu *et al.*, 2016; Imlay, 2003). In this study, I found that GluR, in conjunction with KatM, plays a critical role in H_2O_2 resistance and bacterial virulence.

Loprasert *et al*. (1996) reported the involvement of bacterial catalases in the H_2O_2 detoxification and increased tolerance to oxidative stress. Here I show that

when *gluR* is mutated, exogenous H_2O_2 resistance and catalase activity are reduced, suggesting that GluR is necessary for *B. glumae* to resist H_2O_2– induced stress. Previously, our lab reported that *B. glumae* BGR1 prevented H_2O_2 toxicity by regulating *katG* catalase gene expression through a QS regulator, QsmR (Chun *et al*., 2009). However, the expression of *katG* in the *gluR* mutant was indistinguishable from that of the wild type, I, therefore, attributed the impaired catalytic activity to GluR's direct regulation of *katM* expression in this study. This result was somewhat predicted, given that *gluR* mutations did not affect *B. glumae* QS. The increased expression of *katE* in this study was not significant to the observed phenotypes of the *gluR* mutant. Since there are no solid statements to justify the importance of *katE* in the H_2O_2 susceptible *gluR* mutant, I suspect that *katE* is either a metabolic bi-product or performs different functions (Moriwaki *et al.*, 2008; Yuan *et al.*, 2021) under the control of GluR.

The involvement of GluR in H_2O_2 degradation is not surprising, as TCSs have been reported to mediate response to oxidative stresses in different bacteria species (Singh, 2000; Chen *et al.*, 2008; Loui *et al.*, 2009). TCSs influence bacterial tolerance to reactive oxygen through a variety of mechanisms, including gene regulation (Loui et al., 2009), stabilization of bacterial stress factors (Tu *et al.*, 2006), and macrophage killing (Chen et al., 2008). Anaerobic conditions are among the TCSs environmental triggers that result in oxidative stress resistance responses (Georgellis et al., 2001). Since a cognate sensor kinase is yet to be identified and *B.*

glumae cells cannot grow under anaerobic conditions (Chun *et al*., 2009), it is unknown whether anaerobic conditions or reactive oxygen species serve as environmental stimuli to the regulatory role of GluR in this study.

Catalase activity has become inducible over time to aid pathogens in colonizing their hosts and causing disease by detoxifying H_2O_2 (Miller & Britigan, 1997). According to Chun et al., *B. glumae's katG* is needed for maximum virulence in the host rice plants. In this chapter, I also show that, in addition to the attenuated catalytic activity *in-vitro*, disease severity was significantly reduced in rice stems infected with the *katM* null mutant, a pattern similar to that seen with the *gluR* mutant. This observation is not unique to *B. glumae*; similar findings have been made in other pathogenic bacteria species (Yu et al., 2016; Jittawuttipoka *et al.*, 2009). Therefore, in addition to our earlier findings of the QS-meditated *katG* effect (Chun et al., 2009), I infer that *B. glumae* employs several regulatory mechanisms to mitigate the toxic effects of reactive oxygen species, thrive, and cause disease to the host plant.

In conclusion, I identified T3SS regulators, GluR and Lon protease, which inversely modulate the virulence of *B. glumae* BGR1 (Fig. 12). Given that Lon protease activates the activities of *gluR* while suppressing a direct T3SS regulator, *hrpB*, and that GluR activates *hrpB* as well as *katM* catalase, I propose that GluR and Lon protease work together to protect *B. glumae* BGR1 from the host immunity and related stresses, culminating in bacterial fitness, survival, and successful

infection. It's worth noting that Lon protease's negative control of T3SS genes in *B. glumae* BGR1 is only noticeable when GluR is present since *gluR* mutation in the *lon* mutant abolished T3SS gene expression. In addition, our previous studies reported QS modulated virulence in *B. glumae* (Chun et al., 2009; Kim et al., 2004; Kim et al., 2007; Goo et al., 2010) although I found no link between QS and TCS. Together, our combined studies provide insights into the regulation of bacterial virulence (Fig. 12), providing diverse cues to design effective antimicrobial drugs for the prevention of *B. glumae.*

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Zhou, T., Yin, C., Zhang, Y., Shi, H., Wang, J., Sun, L.*, et al.* (2016). Lon protease is involved in RhpRS-mediated regulation of type III secretion in *Pseudomonas syringae*. *Mol. Plant Microb. Interact.* 29, 807-814. doi: 10.1094/MPMI-06-16-0114-R.

Zhou, X., Teper, D., Andrade, M. O., Zhang, T., Chen, S., Song, W.-Y.*, et al.* (2018). A phosphorylation switch on Lon protease regulates bacterial type III secretion system in host. *MBio,* 9. doi: 10.1128/mBio.02146-17.

Zhou-qi, C., Bo, Z., Guan-lin, X., Bin, L. and Shi-wen, H. (2016). Research status and prospect of *Burkholderia glumae*, the pathogen causing bacterial panicle blight. *Rice Sci.* 23, 111-118. doi: 10.1016/j.rsci.2016.01.007.

Table 1. Bacteria strains that were used in this study.

Strain or plasmid	Characteristics	Source
Burkholderia glumae		
BKATM	BGR1, *katM*::Tn*3*-*gusA21*, Km^R	This study
BKATMC	BGR1, *katM*::Tn*3*-*gusA21* containing pBGM1, Km^R, Tet^R	This study
BLONN	BGR1, *lon*::gm; colony type 2, Gm^R	Our lab
BLONC	BGR1, lon::gm carrying pLa4, Gm^R, Tet^R	Our lab
BGLULON	BLONN, carrying pBGH13, Gm^R, Km^R	This study
Plasmid		
pBGM1	Cosmid clone containing *katM* gene cloned in pLAFR3 Tet^R	This study
pBGM2	3.2 kb pBGM1 *Bam*H1-*Hin*dIII fragment containing *katM* gene, cloned into pLAFR3, Tet^R	This study
pLa4	2.9 kb pLa3 fragment cloned into *Hind*III and *Eco*R1 sites in pLAFR3, Tet^R	Our lab
pLon-His	*lon* in pRT21b, Amp^R	Our lab
pHrpB-His	*hrpB* in pRT21b, AmpR	Our lab

Rif^R rifampicin resistance; Tet^R, tetracycline resistance; Km^R, Kanamycin resistance; Amp^R, ampicillin resistance; Gm^R, Gentamicin resistance

Table 2. List of primers (Source, Macrogen, Korea)

Primers	**Sequence (5' to 3')**
HrpB-F	TGCGCCTATCTCGAACGGA
HrpB-R	AGAGATATTCGAGCTGGCGA
HrpG-F	CGATTCTCCCGTCAGTTCCA
HrpG-R	CCTATACGCTCGACCAGCA
HrcC-F	ACCAACTCGGTGCTGATCC
HrcC-R	AGCGTGTTGTCGTCGATCTC
GluR-F	CAAACACCTTCAGCACCGAG
GluR-R	CGGACGATTACCTGCCCAAG
KatM-F	GACATCGCCACCGAAGAACT
KatM-R	TAAAGCTCGGCCTGCGTATC
KatE-F	AACCATGTGACGAACGACGA
KatE-R	TACATATCGCTGAGCGTGGC
KatB-F	GGAGGAGATTCCGGTCGAGA
KatB-R	GACTTCTGCGTGTCGAGGTA
KatG-F	TCTTCTGGAACCTGTTCGGC
KatG-R	TCTTTTCGTAGGCCGGATCG
KatS-F	ACATCAACTACGACCCGACG
KatS-R	CAGCTTGTACGAATCGGCAT
KatQ-F	ACTTCGTGATGGTCAATGCG
KatQ-R	AGGTGTAGAAGGTCTCGCCG
hrpBp-F	TGAGCGCGTTCACCTCGCAA
hrpBp-R	ATGAGGCGACTCTCCTTGCT
katMp-F	CATTTGCGCAGGATAACCAA

katMp-R	TTGTGCAGATGGAACAGGAC
gluRp-F	ACCTCTGGTACTTGAACGAAC
gluRp-R	TCCATGGGCGGCATCTTATCG

F- forward primer; R – reverse primer

Fig. 1. The role of GluS-GluR TCS in the virulence of *B. glumae* BGR1. (**A**) Response of nonhost tobacco to infection with the wild-type BGR1, TCS mutants (BGLUS35 and BGLUR133), *gluR* mutant complemented strain (BGLUR133C), and distilled water (SDW). (**B-C**) Disease symptoms (**B**) and bacterial growth (**C**) of the indicated *B. glumae* strains in the host rice plants. . At a three-day interval post-inoculation, disease symptoms were photographed, disease index was scored by ImageJ software using the wild type as the baseline (the numbers below the diseased rice stems). The bacteria cells were recovered by crushing the stems in distilled water, plated for CFU count and expressed as Logarithm of CFU/stem. The statistical analysis was performed using one-way ANOVA followed by Tukey's correlation for multiple comparisons. Superscripts (a and b) before the mean value indicate statistical significances, $p < 0.05$; ($F(8,11) = 22.047$; $p = 0.00$). Data represent the mean ± standard error (SE) of triplicates.

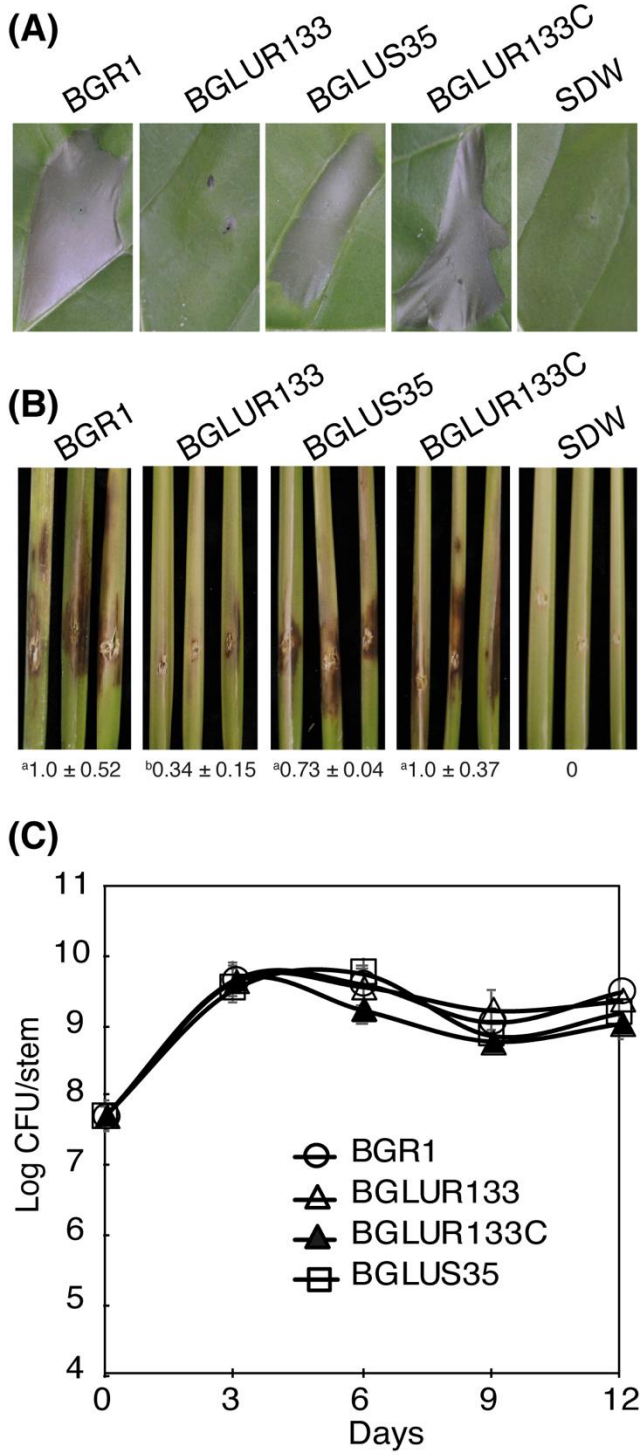

(A)
BGR1
BGLUR133
BGLUS35
BGLUR133C
SDW
(B)
BGR1
BGLUR133
BGLUS35
BGLUR133C
SDW
a1.0 ± 0.52
b0.34 ± 0.15
a0.73 ± 0.04
a1.0 ± 0.37
0
(C)
Log CFU/stem
11
10
9
8
7
6
5
4
0
3
6
9
12
Days
BGR1
BGLUR133
BGLUR133C
BGLUS35

Fig. 2. GluS-GluR TCS has no significant effect on the production and regulation of toxoflavins in *B. glumae.* (**A**) DMSO solutions of the toxoflavins extracts from the wild type and TCS mutants were analyzed on a silica gel coated TLC plate at 365 nm UV light. (**B**) A bioassay of *N*-acyl homoserine lactone (HSL) on *Chromobacterium violaceum* agar plates showed that all the *B. glumae* strains produced comparable HSL signals. 1, BGR1; 2, BGLUS35; 3, BGLUR133.

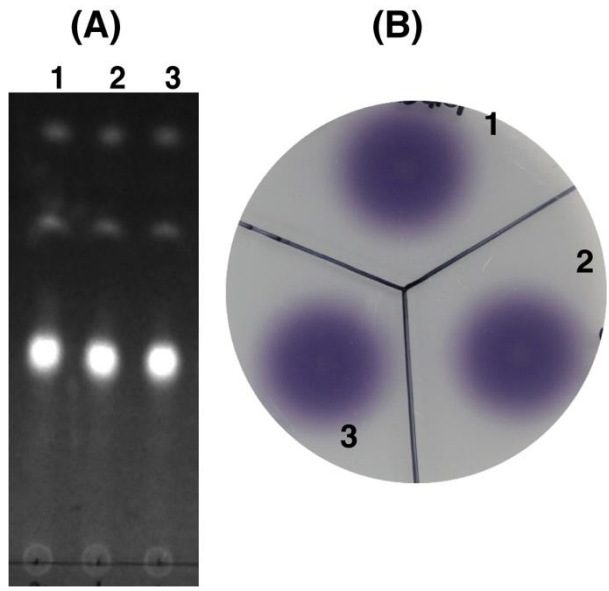


Fig. 3. GluR regulates cell division and pathogenicity in *B. glumae* separately. While constitutive expression of ftsA gene in *gluR* resulted in normal cell division (chapter 1), HR response in tobacco leaves remained unaffected.

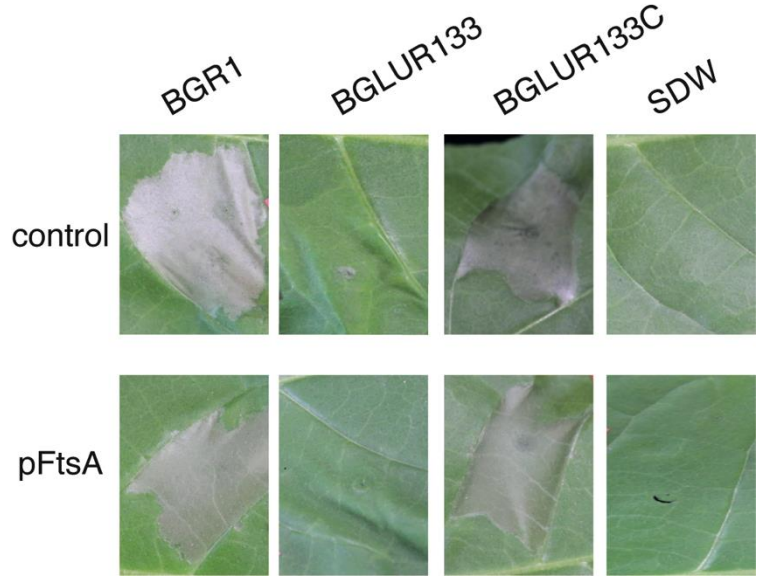


Fig. 4. Impact of *gluR* and *lon* mutations on the T3SS gene induction and pathogenicity of *B. glumae.* (**A**)The bacteria strains were infiltrated into tobacco plants and photographs were taken after 24 hours. (**B**) Real-time PCR showing the expression of T3SS genes (*hrpB, hrcC,* and *hrpG*) in *B. glumae* strains in LB medium using 16S *rRNA* as a reference gene. 1, BGR1; 2, BGLUR133; 3, BGLUR133C; 4, *lon* mutant BLONN; 5, *lon* mutant complementation (BLONC); 6, *gluR/lon* double mutant (BGLULON); 7, *lon* mutant complemented in the *gluR/lon* double mutant (BGLULON (pLA4)); 8, *gluR* mutant complemented in the *gluR/lon* double mutant (BGLULON (pBGH13)); 9, distilled water.

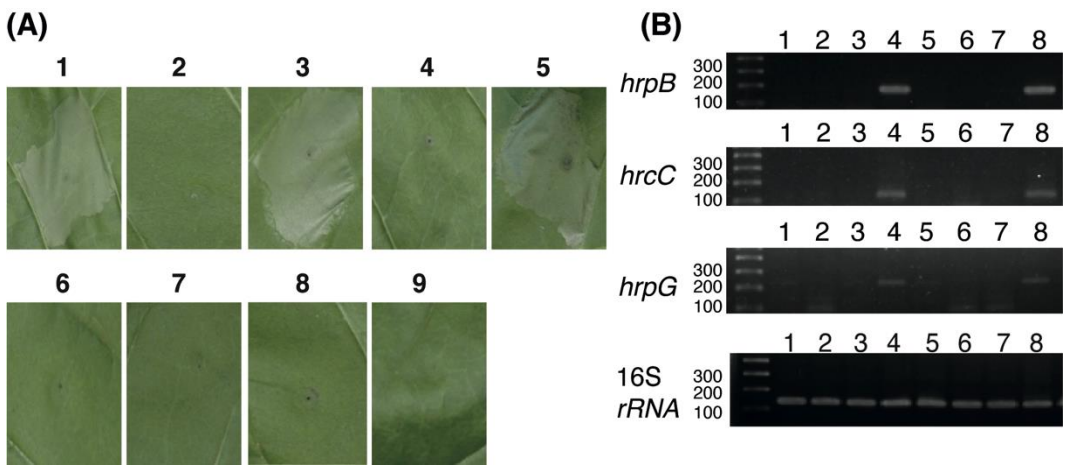
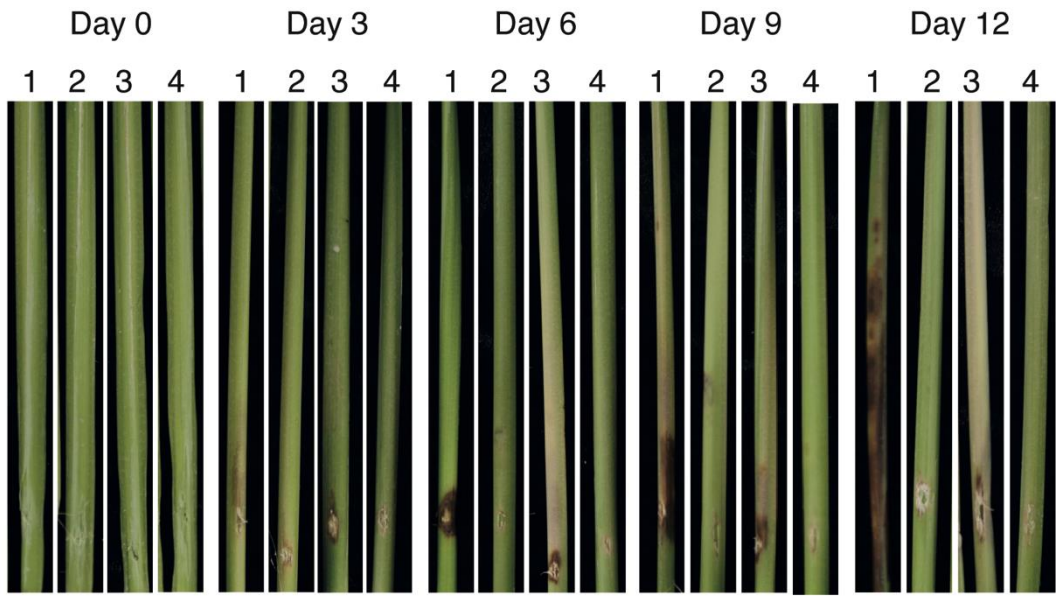


Fig. 5. Response of *lon* mutation in host rice plants. (**A**) Disease symptoms of the *B. glumae* strains in rice plants photographed at a three-day interval. (**B**) Quantification of bacteria cells recovered from the infected rice stems, expressed as log CFU/stem. Data represent the mean ± standard error (SE) of triplicates. 1, BGR1; 2, *lon* mutant BLONN; 3, *lon* mutant complementation (BLONC); 4, distilled water.

(A)


Day 0
Day 3
Day 6
Day 9
Day 12
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

(B)

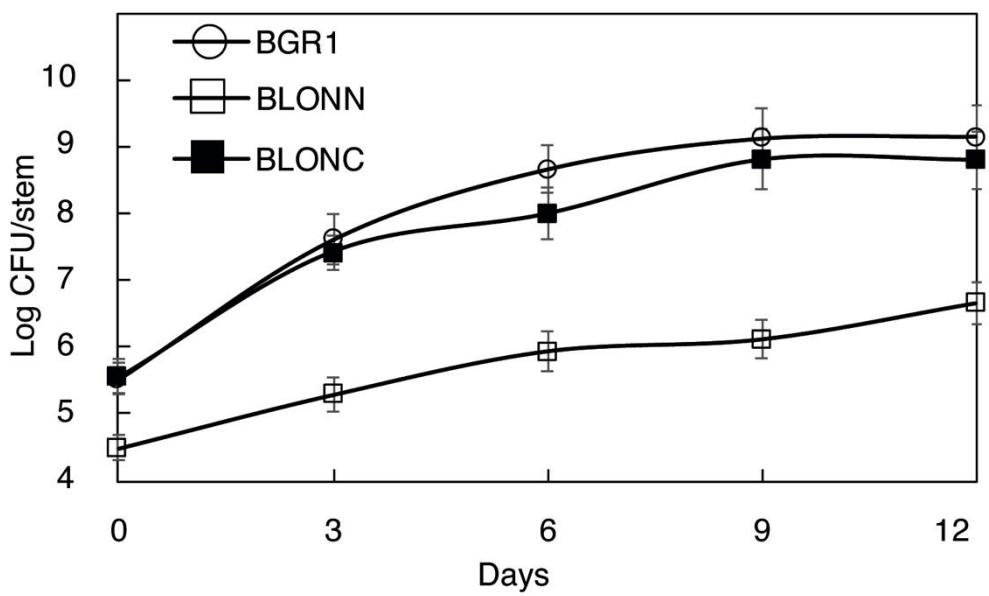

BGR1
BLONN
BLONC
Log CFU/stem
10
9
8
7
6
5
4
0
3
6
9
12
Days

Fig. 6. GluR regulates T3SS of *B. glumae* in an *in-vivo* environment. (A) Real-time – PCR showing the expression of *hrpB* and *hrpG* in 1, wild-type BGR1; 2, *gluR* mutant (BGLUR133); and 3, *gluR* mutant complementation (BGLUR133C). The bacteria strains were cultured in LB medium supplemented with tobacco plant extracts to mimic plant apoplast conditions. (**B-C**) Electrophoretic mobility shift assay (EMSA). (**B**) Gene map showing the putative promoter region of *hrpB*. (**C**) Competition binding assay reaction contained: 1 µM GluR-His, 1 nM labeled *hrpB* DNA, 1 nM unlabeled *katE* non-competitor DNA, and 20 nM unlabeled *hrpB* promoter DNA. Reaction i, GluR-His; ii, labeled *hrpB* DNA*;* iii, unlabeled *hrpB* DNA; iv, unlabeled *katE* DNA, v, GluR-His + *hrpB*; vi, free *hrpB* DNA.

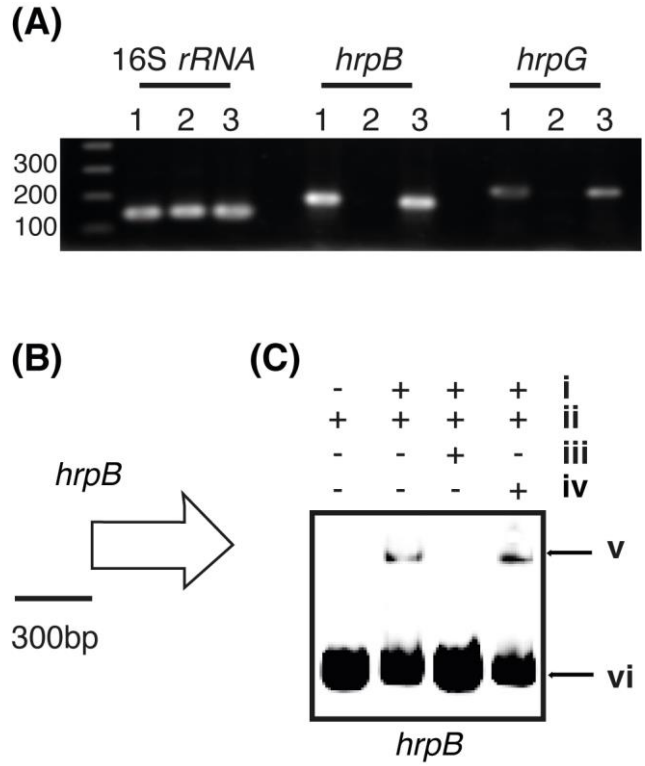
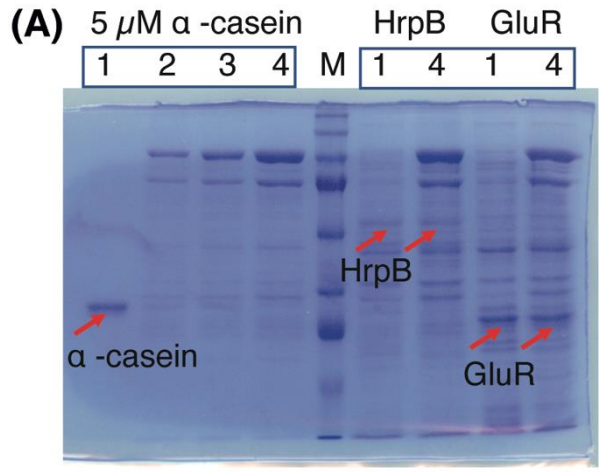

(A)
16S rRNA
hrpB
hrpG
1 2 3 1 2 3 1 2 3
300
200
100
(B)
hrpB
300bp
(C)
- + + + i
+ + + + ii
- - + - iii
- - - + iv
v
vi
hrpB

Fig. 7. Lon protease interacts with both *gluR* and *hrpB* but, neither is degraded.

(A) *In-vitro* proteolysis assay. Purified His-tagged Lon in concentrations of 0, 5, 10, 20 µM (reactions 1, 2, 3, 4 respectively) was incubated with 5 µg cell lysate (HrpB, GluR induced cell) at 37℃ for 1h in the presence of 10 mM ATP. α-casein was used as a control substrate. Samples were analyzed on 10% SDS-PAGE, followed by Coomassie staining. The respective protein bands are labeled with arrows. (**B**) qRT- PCR showing the expression of *gluR* gene in the *lon* mutant (BLONN) and complemented *lon* mutant (BLONC) normalized to that of BGR1. **(C)** Electrophoretic mobility shift assay (EMSA) showing direct binding of Lon-His to the putative promoter regions of *gluR* and *hrpB*. Competition assay reaction between Lon-His and *gluR* contained: 0.5 µM Lon-His, 1 nM labeled *gluR* DNA, 1 nM unlabeled *katE* non-competitor DNA, and 50 nM unlabeled *gluR* DNA. Lon-His and *hrpB* reaction contained: 0.5 µM Lon-His, 0.5 nM labeled *hrpB* DNA, 0.5 nM unlabeled *katE* non-competitor DNA, and 25 nM unlabeled *hrpB* DNA. Reaction i, Lon-His; ii, labeled *gluR* or *hrpB* DNA*;* iii, unlabeled *gluR* or *hrpB* DNA; iv, unlabeled *katE* DNA, v, Lon-His + *gluR* or *hrpB*; vi, free *gluR* or *hrpB* DNA.


(A)
5 μM α -casein
HrpB
GluR
1 2 3 4
M
1 4 1 4
HrpB
α -casein
GluR

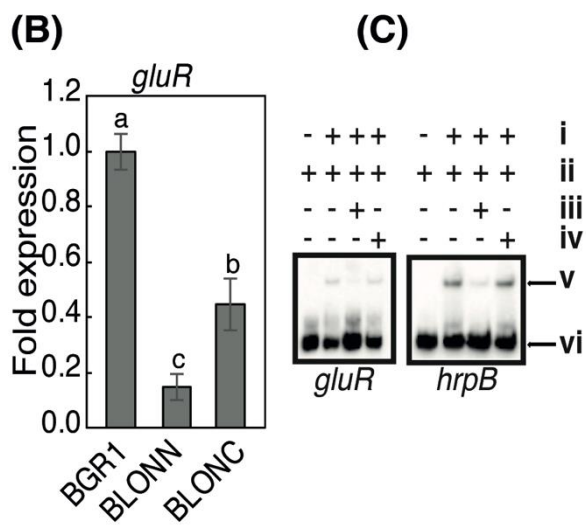
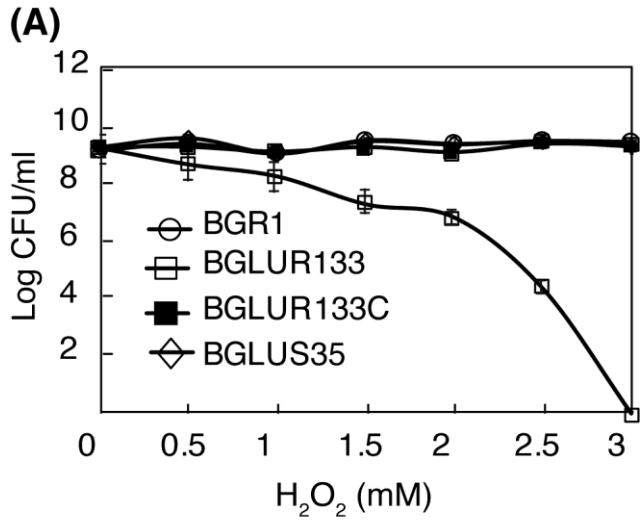

(B)
gluR
Fold expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
BGR1
BLONN
BLONC
(C)
- + + +
+ + + +
- - + -
- - - +
- + + +
+ + + +
- - + -
- - - +
i
ii
iii
iv
v
vi
gluR
hrpB

Fig. 8. GluR improves bacterial viability under H_2O_2. (**A**) H_2O_2 sensitivity assay of the wild-type BGR1, TCS mutants (BGLUS35 and BGLUR133) and *gluR* complemented BGLUR133C. The bacteria strains were cultured in LB medium containing varying concentrations of H_2O_2 as indicated and the population density expressed as log CFU/ml. (**B**) Catalase activity assay. Catalase activity associated with the cell extracts was measured at 240 nm. One unit (U) is defined as the amount of activity required to decompose 1µmol of 1M H_2O_2. The data represents the mean ± standard error (SE) of experiments performed in triplicates. Letters a, b, and c indicate statistical significances, $p < 0.05$; ($F(8, 11) = 22.047$; $p = 0.00$).


(A)
12
10
8
6
4
2
Log CFU/ml
BGR1
BGLUR133
BGLUR133C
BGLUS35
0
0.5
1
1.5
2
2.5
3
H2O2 (mM)

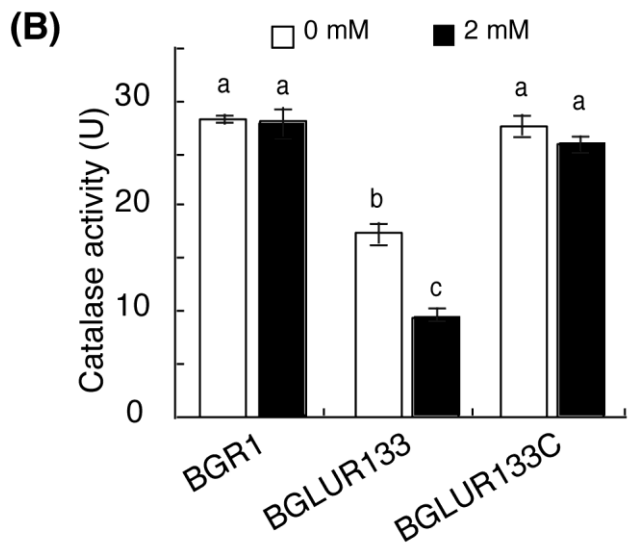

(B)
0 mM
2 mM
Catalase activity (U)
30
20
10
0
a
a
b
c
a
a
BGR1
BGLUR133
BGLUR133C

Fig. 9. GluR positively regulate the expression of *katM* in *B. glumae*. (**A**) Catalase gene expression in BGLUR133 and BGLUR133C normalized to that of the wild-type BGR1. RT-qPCR showed a significant reduction in the expression of *katM* gene in the *gluR* mutant BGLUR133 (red box). Letters a and b show statistical significance, $p < 0.05$; ($F(2, 8) = 5288.133$; $p = 0.00$). the data represent the mean ± standard error (SE) of triplicates. (**B**) Gene map showing the putative promoter region of *katM* for EMSA. **(C)** EMSA showing direct binding of GluR-His to the putative promoter region *katM*; 1 µM GluR-His, 1 nM labeled target DNA, 1 nM unlabeled *katE* non-competitor DNA, and 20 nM unlabeled target promoter DNA was used for EMSA. i, GluR-His; ii, labeled *katM* DNA*;* iii, unlabeled *katM* DNA; iv, unlabeled *katE* non-competitor DNA, v, GluR-His + *katM*; vi, free *katM* DNA.

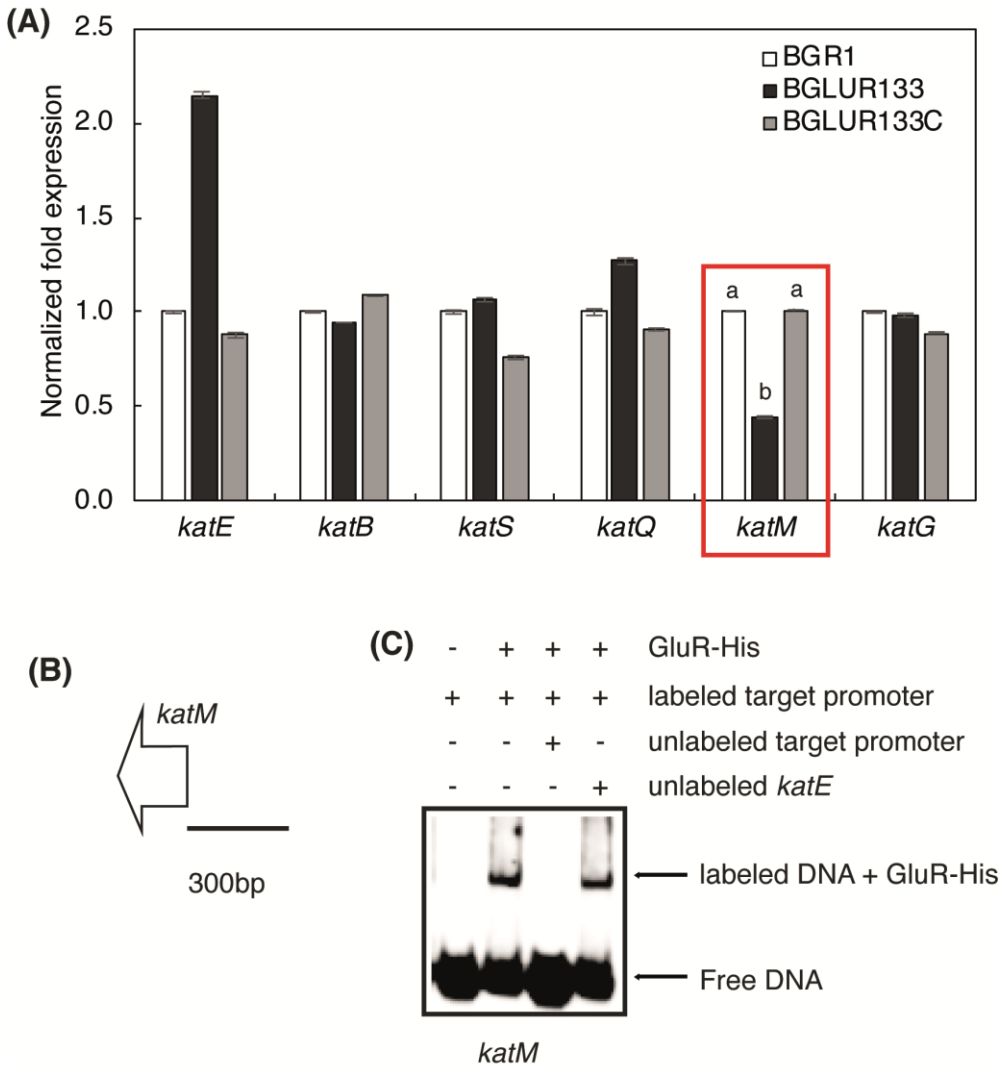

(A)
Normalized fold expression
2.5
2.0
1.5
1.0
0.5
0.0
BGR1
BGLUR133
BGLUR133C
a
b
a
katE
katB
katS
katQ
katM
katG
(B)
katM
300bp
(C)
- + + + GluR-His
+ + + + labeled target promoter
- - + - unlabeled target promoter
- - - + unlabeled katE
labeled DNA + GluR-His
Free DNA
katM

Fig. 10. *katM* and its catalytic activity. (**A**) Organization scheme of *katM* in pBGM1 in *B. glumae* BGR1. Vertical bars above the restriction map indicate the position and direction of the Tn*3*-*gusA* insertions (21). E, *Eco*RI; N, *Not*1; H, *Hin*dIII; B, *Bam*HI; Kp, *Kpn*1. (**B**) Catalase activity of BGR1, BGLUR133, *katM* mutant BKATM, and the respective mutant complemented strains, BGLUR133C and BKATMC measured at 240 nm. One unit (U) is defined as the amount of activity required to decompose 1µmol of 1M H_2O_2. The data represents the mean ± standard error (SE) of experiments performed in triplicates. Letters a, b, c, d, and e indicate statistical significances, $p < 0.05$; ($F (9, 29) = 80.320$; $p = 0.000$).

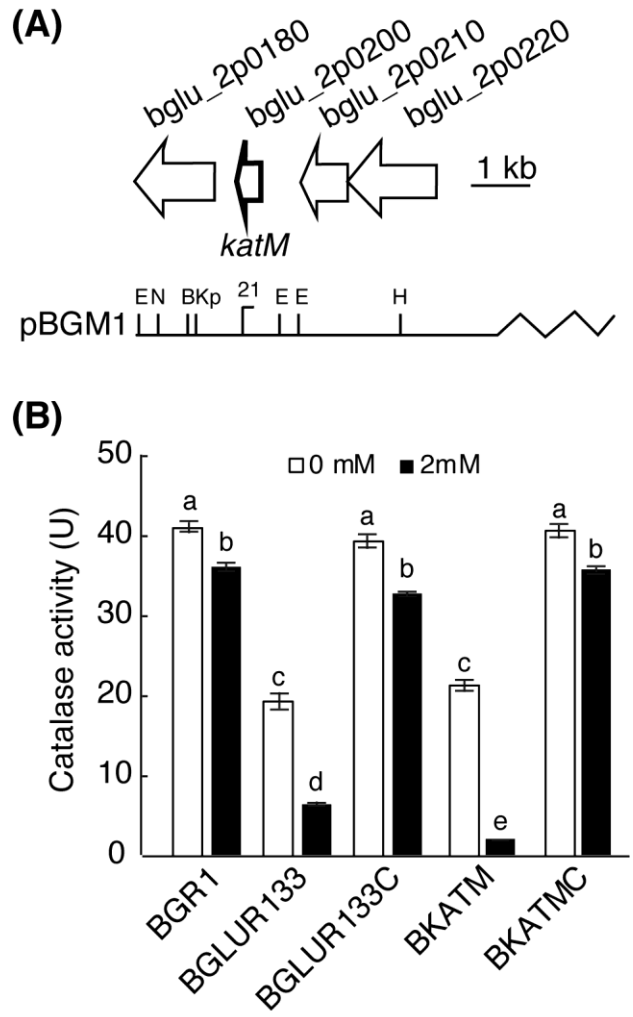
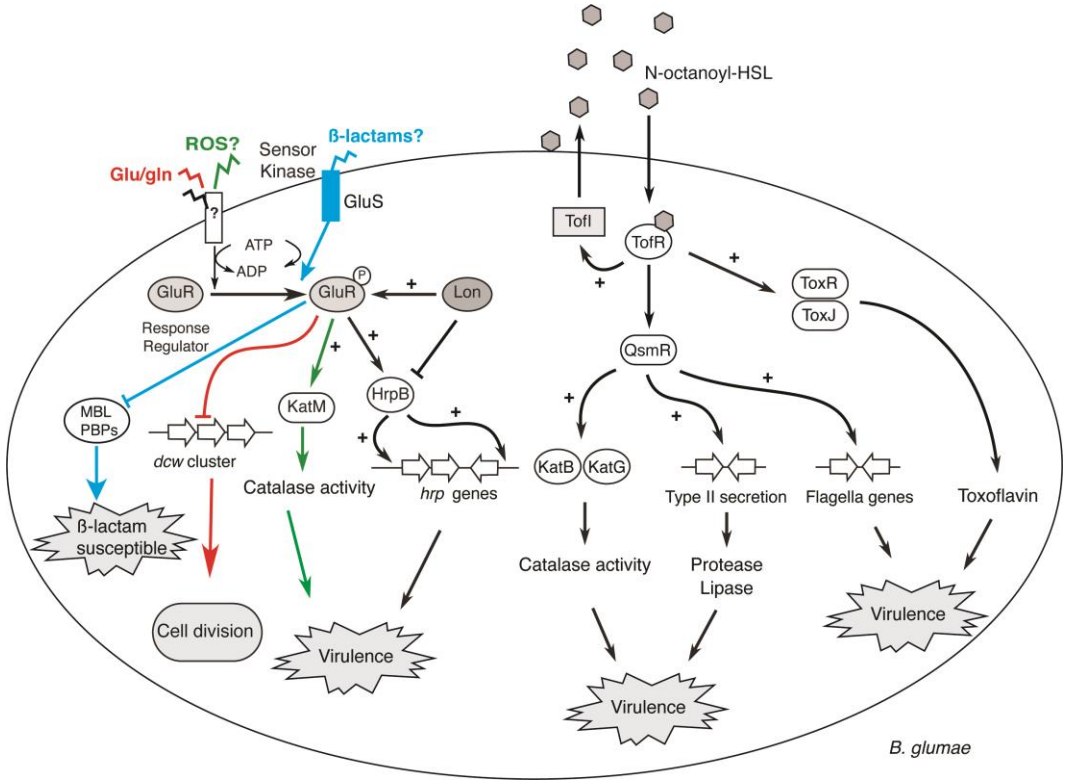

(A)
bglu_2p0180
bglu_2p0200
bglu_2p0210
bglu_2p0220
1 kb
katM
EN BKp 21 E E H
pBGM1
(B)
0 mM
2mM
Catalase activity (U)
50
40
30
20
10
0
a
b
c
d
a
b
c
e
a
b
BGR1
BGLUR133
BGLUR133C
BKATM
BKATMC

Fig. 11. *katM* and the associated role in the virulence of *B. glumae*. Disease symptoms in stems infected with *B. glumae* strains scored in comparison to the wild type. The scale used, zero denotes no symptoms and one indicates symptoms from the wild type. Photographs were taken at 7 days post-inoculation and disease symptoms were disease index scored using ImageJ software (numbers below the diseased rice stems). Superscripts (a, b, and c) before the mean value indicate statistical significances.

Fig. 12. Schematic representation this dissertation and virulence signaling cascades in *B. glumae* BGR1. The regulatory pathways are proposed based on our current observation and our previous studies. The red and blue pathways summarize chapter 1 and chapter 2, respectively. Chapter 3, the present study demonstrated the inverse regulatory functions of TCS response regulator -GluR and Lon protease. A yet-unknown sensor kinase phosphorylates GluR (p), which then positively regulates AraC-type transcriptional activator *hrpB*, and *katM* catalase promoting the respective cellular responses. In addition to activating GluR, Lon protease also directly represses *hrpB*, shutting off the *hrp* gene cascade. Our previous studies reported TofI-tofR QS, which positively modulates virulence by interacting with diverse regulators. + stands for activator; ? stands for unknow/not confirmed; flattened arrow means repressor; and p means phosphorylated.


N-octanoyl-HSL
ROS?
Glu/gln
Sensor Kinase
ß-lactams?
GluS
?
ATP
ADP
GluR
GluR
P
Lon
Response Regulator
TofI
TofR
ToxR
ToxJ
QsmR
MBL PBPs
dcw cluster
KatM
HrpB
Catalase activity
hrp genes
KatB
KatG
Type II secretion
Flagella genes
Toxoflavin
ß-lactam susceptible
Cell division
Virulence
Catalase activity
Protease Lipase
Virulence
Virulence
B. glumae

APPENDICES

Appendix 1. Similarities among two-component system protein sequences computed by ClustalW multiple sequence alignment using the BioEdit sequence alignment editor. (**A**) Comparison between the GluR response regulator of *B. glumae* BGR1 (BGLU_1G13360), BURPS305_7006 of *B. pseudomallei* 305, RisA BMA10247_1253 of *B. mallei* NCTC 10247, and BCENMCO3_1962 of *B. cenocepacia* MCO-3. (**B**) Comparison between the GluS sensor kinase of *B. glumae* BGR1 (BGLU_1G13350), Envz1 BGL_1C23830 of *B. plantarii*, BGLA_1G24110 of *B. gladioli* BSR3, and RisS BMA1486 of *B. mallei* ATCC 23344.

(A) Two-component system Response regulators

```
BGLU_1G13360   MPPMETKNPS KILVVDDDPR LRDLLRRYLG EQGFNVYVAE NATAMNKLWV RERFDLLVLD
BURPS305_7006  MPLMETKNPS KILVVDDDPR LRDLLRRYLG EQGFNVYVAE NATAMNKLWV RERFDLLVLD
BMA10247_1253  MPLMETKNPS KILVVDDDPR LRDLLRRYLG EQGFNVYVAE NATAMNKLWV RERFDLLVLD
BCENMCO3_1962  MPIMETKNPS KILVVDDDPR LRDLLRRYLG EQGFNVYVAE NATAMNKLWV RERFDLLVLD

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  65         75         85         95        105        115
BGLU_1G13360   LMLPGEDGLS ICRRLRGSND RTPIIMLTAK GEDVDRIVGL EMGADDYLPK PFNPRELVAR
BURPS305_7006  LMLPGEDGLS ICRRLRGSND RTPIIMLTAK GEDVDRIVGL EMGADDYLPK PFNPRELVAR
BMA10247_1253  LMLPGEDGLS ICRRLRGSND RTPIIMLTAK GEDVDRIVGL EMGADDYLPK PFNPRELVAR
BCENMCO3_1962  LMLPGEDGLS ICRRLRGSND RTPIIMLTAK GEDVDRIVGL EMGADDYLPK PFNPRELVAR

               ....
```

(B) Two-component system Sensor kinases

```
BGLU_IG13350   MRIDRRLLTL VFGGLFWRTF LLIALLIAVS LAAWFQSFRV IEREPRAQRV ALQLVAVVKL
BGL_1C23830    MRIDRRLLTL VFGGLFWRTF LLIALLIAVS LAAWFQSFRV IEREPRAQRV ALQLVAVVKL
BGLA_1G24110   MRIDRRLLTL VFGGLFWRTF LLIALLIAVS LAAWFQSFRV IEREPRAQRV ALQLVAVVKL
BMA1486        MRIDRRLLTL VFGGLFWRTF LLIALLIAVS LAAWFQSFRV IEREPRAQRV ALQLVAIVKL

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  65         75         85         95        105        115
BGLU_IG13350   TRTALLYSDP DLRRALLQDL ESNEGVRVYP RESTDKFKLQ PDESLNRLIE HDIRSRLGDD
BGL_1C23830    TRTALLYSDP DLRRALLQDL ESNEGVRVYP RETTDKFKLQ PDESLNRLIE HDIRSRLGDD
BGLA_1G24110   TRTALLYSDP DLRRALLQDL ESNEGVRVYP RETTDKFKLQ PDESLNRLIE HDIRSRLGDD
BMA1486        TRTALLYSDP DLRRALLQDL ESNEGVRVYP REKTDKFKLQ PDESVNRLIE HDIRSRLGDD

               ...
```

세균성벼알마름병원균의 GluS-GluR Two-Component System 의 기능 연구

마룬가 조안

Burkholderia glumae 는 다양한 미생물 유기체들이 어떻게 다양한 환경에서 진화, 적응하는지에 대한 통찰력을 제공하는 다양한 생물학적 기능 시스템들을 가지고 있다. 이러한 시스템들에 대한 일부의 복잡성에도 불구하고 본 연구는 일반적인 세균에서의 정보처리 흐름의 기초적 역할을 담당하는 two-component regulatory systems (TCS)의 패러다임을 제시하고자 한다. mini-Tn5 를 사용한 *B. glumae* BGR1 의 mini-Tn5 무작위 돌연변이 유도체 중 하나는 Luria–Bertani (LB) 배지에서 필라멘트 모양의 세포형태로 발견되었다. 이 돌연변이 유도체에 대한 분자 및 유전적 분석은 이것이 two-component regulatory systems 반응 조절 유전자인 *gluR* (BGLU 1G13360)에 mini-Tn5 삽입 돌연변이를 가지고 있음을 밝혔다. 이 *gluR* 의 전사방향 아래에서 TCS 감지-인산화효소인 *gluS* (BGLU 1G13350)가 발견되어 B. *glumae* BGR1 의 *GluS-GluR* TCS 의 기능과 역할을 추적할 수 있게 되었다. *gluR* 돌연변이는

LB 배지에서 필라멘트 세포를 형성한 *gluS* 돌연변이와 달리 42°C 에 민감하며, 세포 분열 및 세포벽 (dcw) 생합성을 담당하는 유전자들의 발현을 wild type 에 비해 증가되었기에 GluR 을 세포 분열의 필수 조절 인자로 파악하였다. TCS 는 감지-인산화효소가 환경 신호를 감지하여 반응 조절기에 전달하여 적절한 세포 반응을 유도하는 체계적인 시스템을 통해 다양한 세균 활동을 조절한다. 이 연구에선 *B. glumae* 에서 GluR 이 세포 분열을 시작하는 외부 신호로 감지하는 것을 글루타민과 글루타메이트로 확인했다. 또한, GluR 은 담배 잎에서 과민성 반응의 유도와, 숙주인 벼에서의 완전한 독성발현 및 식물의 방어기작인 과산화수소의 해독을 위해 필요했다. 이 모든 것은 *B. glumae* 의 병원성, 생존 및 환경적응의 중요한 요소들에 GluR 이 관여하는 것이다. GluR 은 III 형 분비 시스템 및 망간 항산화효소 유전자 *katM* 과 직접 상호 작용하여 병원균의 독성 및 병원성을 촉진한다. 이 연구에서는 GluS-GluR 이 *B. glumae* 의 β- 락탐 항생제 내성을 조절하는 것에 기능적으로 연결되어 있으나, 서로 구별되는 메커니즘을 통해 항생제 내성이 만들어짐을 추가로 보여주었다. *gluS* 또는 *gluR* 의 비활성화는 β-lactam 항생제에 대한 내성을 부여한 반면, wild type 은 이러한 항생제에 민감하였다. 이러한 표현형은 wild type 에

비해 TCS 돌연변이체에서 β-락탐 분해효소 및 페니실린 결합 단백질을 코딩하는 유전자들의 발현이 현저하게 증가된 것에 뒷받침된다. 전반적으로, 본 연구는 TCS 가 세균의 정교한 조절 시스템에 어떻게 영향을 미치는지에 대한 이해를 더하고, 항생제 내성 반응에 대한 새로운 관점을 제공하며, 병원성 세균의 성공적인 제어를 위한 새로운 치료 방법을 제공 할 수 있다.

Keywords: *Burkholderia glumae*, two-component system, GluR, GluS, cell division, virulence, β –lactam resistance, type III secretion system

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