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Characterization of a σ54-Dependent Response Regulator, tepR, in the Rice-Pathogenic Bacterium Burkholderia Glumae and Development of Biocontrol Strategies for Bacterial Panicle Blight of Rice

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CHARACTERIZATION OF A σ^{54}-DEPENDENT RESPONSE REGULATOR, *tepR*, IN THE RICE-PATHOGENIC BACTERIUM *BURKHOLDERIA GLUMAE* AND DEVELOPMENT OF BIOCONTROL STRATEGIES FOR BACTERIAL PANICLE BLIGHT OF RICE

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DEDICATION

I would like to dedicate this thesis to my parents Mr. Arjun Prasad Osti and Mrs. Kamala Osti for their warm love and great support to be here, and to my sister Mrs. Shobha Osti for her encouragement.

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ABSTRACT

Bacterial panicle blight, caused by *Burkholderia glumae*, is a major bacterial disease of rice in Louisiana. This bacterium contains several virulence factors required for disease development such as toxoflavin, lipase and flagella. In a genome-wide search for regulatory factors related to the virulence of *B. glumae*, *tepR* was identified as a negative regulator for toxoflavin production and found to encode a sigma 54-dependent response regulator. TepR is homologous to LuxO, a quorum-sensing signaling component of *Vibrio* spp. A markerless *tepR* deletion mutant of *B. glumae* 336gr-1, LSUPB401, produced more toxoflavin and showed higher lipase and protease activities compared to the wild type, 336gr-1. The phenotype of mutant LSUPB401 was complemented by a functional *tepR* clone, confirming that *tepR* is a novel negative regulator for toxoflavin production in *B. glumae*. In addition, LSUPB401 was more aggressive than the wild type in causing symptoms on rice panicles and onion bulb scales. However, LSUPB401 lost flagellar motility and hypersensitive reaction, suggesting positive roles of *tepR* in those phenotypes. These findings suggest that TepR promotes the cellular functions for initial host colonization.

Ten bacterial strains showing antagonistic activities against *B. glumae* in an *in vitro* assay were detected from several thousand bacterial isolates obtained from the root region of rice grown at the Rice Research Station, Crowley, Louisiana. Those bacteria were identified as *Bacillus, Paenibacillus* and *Pseudomonas* based on the 16S rDNA sequences. Some isolates suppressed bacterial panicle blight significantly in both greenhouse and field conditions, therefore, are potential candidates for further biocontrol studies.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Rice and bacterial panicle blight

Rice (*Oryza sativa*) is an important cereal crop in the world and about half of total population in the world consumes it as a staple food. Rice is grown in tropical and subtropical regions of the world. Southeastern region of Asia has a suitable environment for rice production and farmers of that area consider rice a major crop. Texas, Louisiana, Arkansas, Missouri, California and Mississippi are the top rice-producing states in the United States. Even though the U.S. only produces 2% of the world's annual rice supply, it is the 3rd largest rice exporter (USA Rice federation, 2013). Diseases and pests are the major detrimental factors that reduce the production of any crop. Rice diseases cause major problems in rice industries in the world. In the United States the major rice diseases are sheath blight caused by *Rhizoctonia solani*; blast caused by *Pyricularia grisea*; brown spot caused by *Cochiobolus miyabeans*; narrow brown spot caused by *Cercospora janseana;* kernel smut caused by *Neovossia horrida* and bacterial panicle blight (Louisiana Rice Production Handbook, 2014).

Bacterial panicle blight is an important disease of rice caused by the bacterial pathogens, *Burkholderia glumae* and *B. gladioli* (Groth and Hollier, 2011). This disease can cause more than 70% loss in yield and milling in rice (Groth and Hollier, 2011). The major pathogen for bacterial panicle blight, *B. glumae*, was first recorded in Japan as a causal organism of grain rot or grain blight of rice (Goto and Ohata, 1956). High humidity and high night-temperature are favorable environmental conditions for *B. glumae* to develop the disease in rice (Nandakumar *et al*., 2008; Tsushima *et al*., 1996). Such weather conditions are found in many tropical regions of the world and the southern part of the United States. In Louisiana, the symptoms of the disease in the rice plant had

been observed for a long time and the symptoms were assumed to be caused by some abiotic factors (Groth, 1991). But, later in 1996/97, it was confirmed that the disease was caused by *B. glumae* and *B. gladioli* (Shahjahan *et al*., 2000). Most of the rice cultivars grown in Louisiana are susceptible to the bacterial panicle blight, but some cultivars and lines, such as LM-1 and Jupiter, show partial resistance (Rush *et al*., 2007). Panicle initiation is the most vulnerable stage of rice growth for the development of bacterial panicle blight. Blighting in panicles and seeds is a typical symptom of this disease but in severe cases whole plant can be killed. Moreover, panicle grains appear to be unfilled and seed coat becomes greyish and straw colored (Ham *et al*., 2011). Significant yield reductions of rice due to outbreaks of bacterial panicle blight were recorded in 1995,1998, 2000 and 2011 in Louisiana (Louisiana Rice Production Handbook, 2014).

1.2 *Burkholderia glumae*

The major pathogen of bacterial panicle blight of rice, *B. glumae*, was previously considered as *Pseudomonas glumae.* Later, *P. glumae* and another rice pathogenic bacterium, *P. plantarii*, were transferred from the genus *Pseudomonas* to *Burkholderia* in 1994 (Urakami *et al*., 1994). *B. glumae* is in a unique bacterial genus with diverse types of species including human pathogens to plant pathogens and non-pathogenic species (Coenye and Vandamme, 2003). *B. glumae* is reported to cause wilts in many field crops including tomato, hot pepper, potato, eggplant, sesame and sunflower (Jeong *et al*., 2003). In the course of disease development, the bacteria infect seeds and invade plumules or infect the plant directly through the stomata or wounds and proliferation occurs in the intercellular spaces of parenchyma (Hikichi *et al*., 1995). *B. glumae* has been reported in major rice growing countries including Japan, China, Korea, the

Philippines, Latin America and the United States (Nandakumar *et al*., 2009). Recently, it has been reported from other rice growing regions of the world including Africa (Zhou, 2014), suggesting that this bacterium is an emerging major pathogen of rice in the world (Ham *et al*., 2011).

B. glumae is a Gram-negative, motile with flagella, aerobic, rod-shaped bacterium. This bacterium can grow between 11- 40 ^{0}C, but optimum growth range is between 30 to 35^{0}C (Brenner *et al*., 2005). In rice *B. glumae* is considered a seed-borne pathogen but this has been also found in soil. Another species of *Burkholderia*, *B. gladioli* also causes bacterial panicle blight in rice (Nandakumar *et al*., 2009) but *B. glumae* is the major one causing bacterial panicle blight. *B. glumae* is closely related to other rice pathogenic species, *B. plantarrii* and *B. gladioli,* but distant from *B. cepacia* which causes sour skin disease in onion (Jacobs *et al*., 2008). Some strains of *B. glumae* can be opportunistic human pathogens being isolated from an infant suffering from granulomatous disease (Weinberg *et al*., 2007).

The major known virulence factors and virulence-related traits of *B. glumae* are toxoflavin, lipase and flagellum-dependent motility (Devescovi *et al*., 2007; Kim *et. al*., 2004; Kim *et al*., 2007). Toxoflavin-deficient and lipase-deficient mutant strains of this bacterium are almost avirulent to rice (Kim *et al*., 2004; Devescovi *et al*., 2007). Toxoflavin, which is considered to be a major disease-causing factor in rice, is a host nonspecific phytotoxin produced by *B. glumae* and a very effective electron carrier generating reactive oxygen species (ROS) (Kim *et al*., 2013; Sato *et al*., 1989). To find out other virulence factors of this bacterium, different genetic approaches are ongoing in plant pathology and microbiology laboratories.

The pathogenicity of *B. glumae* is governed by multiple virulence factors, which are regulated by a global regulatory quorum-sensing (QS) system mediated by the LuxI homologue, TofI and the LuxR homologoue, TofR (Francis *et al*., 2013; Kim *et al*., 2007, 2009). The bacteria utilize QS as a part of their colonization and invasion strategies (Devescovi *et al*., 2007; Kim *et al*., 2004; Kim *et al*., 2007). QS is a cell-to-cell communication process in which bacteria regulate gene expression according to their population density. In most gram-negative bacteria, the QS system is mediated by synthesis of and response to of *N*-acyl homoserine lactones (AHLs) (Netotea *et al*., 2009). In LuxI/LuxR QS system, AHL production is catalyzed by AHL synthase, which belongs to LuxI-family proteins. AHL synthase requires *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (ACP) as a substrate from the fatty acid biosynthesis pathway (Choudhary *et al*., 2013). AHL molecules bind to the sensor/regulator protein belonging to LuxR-family proteins (Figure 1.1). The AHL-binding domain is present in the *N*- terminal region of LuxR, which facilitates the formation of functional homodimers that bind the specific target gene promoters and activate the transcription of those genes (Miller and Bassler, 2001).

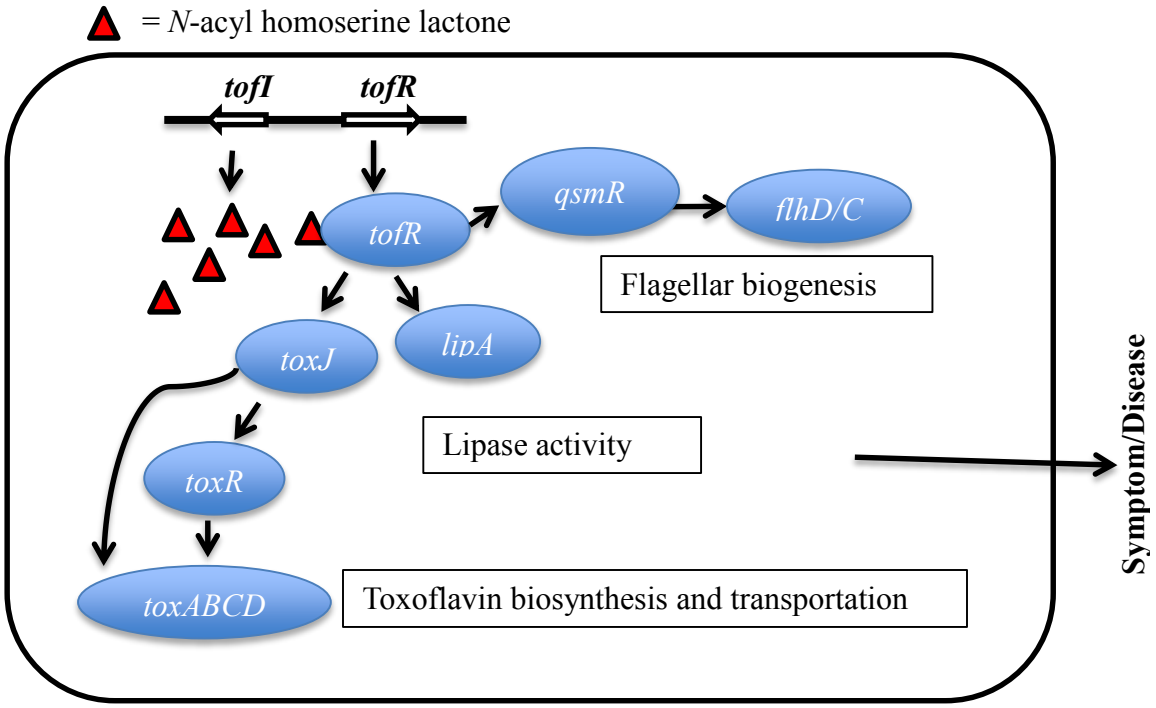

Figure 1.1. A schematic view of TofI/TofR quorum-sensing system for the virulence of *B. glumae* where this system controls the production of known major virulence factors: toxoflavin (Kim *et al*., 2004), lipase (Devescovi *et a*l., 2007) and flagella (Kim *et al*., 2007).

The major regulatory mechanism to control the production of virulence factors of *B. glumae* is QS, which is mediated by LuxI/LuxR homologues TofI/TofR (Figure 1.1) and AHL type signal molecule *N*-octanoyl-L-homoserine lactone (Duerkop *et al*., 2007; Kim *et al*., 2007). TofI also produces another signaling molecule, *N*-hexanoyl-L-homoserine lactone but the function of this molecule is unknown in *B. glumae* and *Burkholderia* spp. (Chen *et al*., 2012).

1.3 Bacterial panicle blight disease management in rice

Judicious application of agrochemicals, development of resistant varieties, improvement on cultural practices, and utilization of biological agents are some important strategies for controlling crop diseases. However, proper management of bacterial diseases of crops is more complicated than fungal diseases. In Japan, oxolinic acid was found to be effective for controlling seedling and grain rot of rice caused by *B. glumae* (Hikichi and Egami, 1995). Unfortunately, the chemical is not registered in the United States and cannot be used in the rice field for the management of bacterial panicle blight (Nandakumar *et al*., 2009). In addition, there is question in the sustainability of this chemical because some strains of *B. glumae* have already developed resistance to oxolinic acid in the rice fields due to mutation (Maeda *et al*., 2004). There are some rice varieties showing different levels of susceptibility and resistance to bacterial panicle blight (Sayler *et al*., 2006). However, no complete resistant variety for bacterial panicle blight has been developed even though partial resistant varieties have been identified (Shahjahan *et al*., 2000). These partial resistant varieties can be used as the genetic sources to develop additional disease resistant varieties through the conventional breeding as well as marker-assisted breeding based on genetic information about partial resistance to bacterial panicle blight (Nandakumar and Rush, 2008).

Biological control studies in rice revealed that some avirulent strains of *Burkholderia* spp. suppressed bacterial panicle blight (Ham *et al*., 2011). An avirulent strain of *Pseudomonas glumae* (which was later renamed as *Burkholderia glumae*), N7503, highly suppressed the bacterial seedling rot of rice when rice seeds were treated by that strain (Furuya *et al*., 1991). There may be many more potential biological control

agents inhabiting the rice plants and its rhizosphere. Some preliminary experiments in our laboratory also suggested that some strains of *Bacillus* spp. isolated from rice plants showed high levels of antagonism against *B. glumae* in *in vitro* assays.

Since, rice is the most important food crop consumed by more than half of the world's population, reduced rice production will negatively affect the global economy (Yuan, 2004). A major bacterial disease of rice, bacterial panicle blight is a potentially serious problem for rice growers because of the seed borne nature of the pathogen, which can be transmitted year-to-year in the field (Trung *et al*., 1993). The severe seed infestation causes reduction of rice yield up to 75% because of sterility on florets and decreased grain quality. Hence, the study of bacterial panicle blight and its major pathogen *B. glumae* is very important to find out the effective control methods of the disease.

CHAPTER 2. CHARACTERIZATION OF σ^{54}-DEPENDENT RESPONSE REGULATOR, *tepR*, IN THE RICE PATHOGENIC BACTERIUM *BURKHOLDERIA GLUMAE*

2.1 Introduction and literature review

2.1.1 σ54-dependent response regulators and their roles in different virulence systems of bacteria

Different regulatory factors ultimately control the ability of a pathogen to cause disease. In pathogenic bacteria, different regulatory genes are involved in maintaining adhesion to colonize on the host surface by controlling the flagellar motility, penetration and proliferation in host tissue via different secretion systems, and destroying the normal cell function through the toxin production (Hao *et al*., 2013).

In bacteria, numbers of cellular activities are regulated by transcription of genes and transcription factors play significant roles to regulate levels of gene expression (Studholme and Dixon, 2003). The regulation of transcriptional initiation, which requires a σ–bound holoenzyme, is a fundamental mechanism for developmental process and adaptation in different environmental conditions for any organism in the living world (Shingler, 1996). Sigma (σ) subunit, which binds RNA polymerases reversibly, is important to bacterial RNA polymerases for the promoter recognition and transcription initiation. A single sigma factor is composed of hundreds of prokaryotic genes and the sigma factor regulates simultaneous expression of those genes, which might contribute to clearly defined primary or multiple physiological functions (Kazmierczak *et al*., 2005). Typical bacterial cells contain different alternative sigma subunits, which have specific sequences and roles to direct RNA polymerase (RNAP) holoenzyme towards different sets of promoters (Studholme and Dixon, 2003). Bacteria contain multiple sigma factors among which the primary sigma factor (σ^{70}) is responsible for transcription of

housekeeping genes necessary for viability and others are referred to as alternative sigma factors, which control specialized functions (Helmann, 2001). Studies have shown that in prokaryotes, mode of transcription initiation mediated by σ^{54} subunit is structurally and functionally distinct from σ^{70} (Merrick, 1993). Different studies were done on σ^{54}-depenent genes in different prokaryotic organisms and found that those genes were responsible for virulence in multiple bacterial species. Sigma-54 is a major central regulator in various pathogenic bacteria and governs multiple cellular processes and virulence traits like motility and biofilm formation (Francke *et al*., 2011). The sigma factor in bacteria controls the biosynthesis and transport of the main precursors of toxins by controlling the related genes in different metabolic and cellular processes (Francke *et al*., 2011). Different studies on σ^{54}-depenent genes on multiple bacterial species reveled that those genes are involved in different virulence systems even though originally it was believed that σ^{54} controls the nitrogen metabolism through transcription of the genes encoding enzymes for nitrogen assimilation and nitrogen fixation (Kazmierczak *et al*., 2005). In bacteria, genes under the control of the σ^{54} factors are regulated by different environmental and metabolic signals (Shingler, 1996).

Different types of genes, which are involved in the survival and adaptation of bacteria under unfavorable environmental conditions, are activated by the alternative sigma factor σ^{54} (RpoN) and ultimately those functions are closely linked to the virulence of pathogenic bacteria (Reitzer and Schneider, 2001). The *rpoN* deletion mutant of *Vibrio anguillarum*, NB10, was defective in flagellum formation and motility, which made the mutant less virulent (O'Toole *et al*., 1997).

In *Pseudomonas aeruginosa*, a *rpoN* mutant did not show any motile activity governed by flagella and cannot synthesize flagellin antigen while wild type showed all of above characteristics; this result reveals that flagellin structural gene is transcribed by RNA polymerase containing RpoN (Totten *et al.,* 1990). Boucher *et al.* (2000) found that σ^{54} represses the *algD* (responsible for the production of alginate) of *P. aeruginosa* in certain environmental conditions. It was found that some sigma factors have critical roles in bacteria-plant interactions which is proved by finding the function of σ^{54} in *P. syringae*, where it controls *hrp* gene expression and influences virulence mechanism (Kazmierczak *et al*., 2005). $HrpL_{Esc}$ is an important alternate sigma factor of the extra-cytoplasmic function family in *Erwinia carotovora* sub sp. *carotovora,* having a vital role for the expression of *hrp* genes, which are responsible for the type III secretion system and production of harpin. Chatterjee *et al.* (2002) found that RpoN (σ^{54}) is required for the expression of $hrpL_{Ecc}$ by demonstrating the lower level of $hrpL_{Ecc}$ transcripts in the $RpoN^{-}$ strain, lacking sigma-54.

In *Vibrio harveyi*, LuxO is homologue of the two-component response regulator protein NtrC (Bassler *et al.*, 1994) and members of this transcriptional activator protein family is composed of a conserved central region containing nucleotide binding and hydrolysis determinants which are required for activating the closed σ^{54} – holoenzyme-promoter complexes (Popham *et al.,* 1989). In several bacteria, σ^{54} is required for the transcription of flagellar biogenesis genes. Lilley and Bassler (2000) performed an experiment to test whether σ^{54} is required for the motility of *V. harvey* by using the soft agar motility plates. They found that the wild type showed swarming, whereas *rpoN::*CM null strain did not show any swarming. In addition, they found that σ^{54} controls the

motility independent of LuxO system. LuxO regulates more than one cellular processes including motility, protease production and biofilm formation in *Vibrio cholera* (Zhu *et al.,* 2002). In *Vibrio anguillarum* M3, Δ*rpoN* mutant lacks swarming motility in soft agar and is deficient in flagellar production (Hao *et al*., 2013). Moreover, the *rpoN* deleted mutant of *V. anguillarum* formed less biofilm and showed significantly lower EPS level as compare to the wild type containing the functional *rpoN* gene, and there was no mortality in fish inoculated with the Δ*rpoN* mutant, whereas 100% mortality was observed with the wild type (Hao *et al*., 2013). There are many evidences pointing out the role of sigma-54 in virulence-related functions in addition to nitrogen assimilation or bacterial metabolism. Iyer and Hancock (2012) tested the effect of *rpoN* (σ^{54}) deletion on extracellular DNA (eDNA) during biofilm development in *Enterococcus faecalis* and observed a lesser amount of eDNA in the biofilm produced by the deletion mutant than by the wild type, suggesting the role of sigma-54 in regulation of the structure and composition of the biofilm matrix. Sigma-54 and its associated activators are also highly important in the field of medicine, food safety and agricultural microbiology because the change in exterior environment affects stability and aggressiveness of deleterious bacterial population (Francke *et al*., 2011).

2.1.2 LuxO type regulatory system

LuxO protein was first characterized in *Vibrio harveyi* as a crucial regulatory component having a role in the quorum sensing system (Bassler *et al*., 1994). In *V. harveyi,* two types of autoinducers, AI-1 and AI-2, are involved in controlling the expression of density-dependent luciferase structural operon *luxCDABE* through the phosphorylation of the response regulator protein LuxO (Bassler, 1999). Mutation on the

quorum-sensing regulator LuxO in *V. cholera* C6706 increased the expression of the gene responsible for type VI secretion system (T6SS) indicating the negative role of LuxO in this important virulence factor (Zheng *et al*., 2010). In general, LuxO-family signal transduction proteins in the bacteria are also referred to as the σ^{54}-enhancer binding protein (Morett and Segovia, 1993). Klose *et al.* (1998) suggested that LuxO acts as a possible repressor or activator at σ^{54} or other σ-dependent promoters in pathogenic bacteria. In functional analysis of a LuxO mutant of the fish pathogen *V. alginolyticus*, it was observed that the mutant produced significantly higher amount of extracellular protease (ECP) in comparison to the wild type strain, suggesting the role of $LuxO_{val}$ as a negative regulation of ECP production (Wang *et al*., 2007). However, in different *Vibrio* spp. the role of LuxO on ECP production might be variable (Wang *et al*., 2007). Zhu *et al*. (2002) observed that a *luxO* mutant of *V. cholera* had a less motility than the wild type in swarm plate and the same mutant was defective in biofilm formation. In *V. harveyi*, Yang and Defoirdt (2014) observed a significantly lower flagellar gene expression and less motility in *luxO* mutant in the background of inactive quorum sensing, than the wild type.

In the QS network of *V. harveyi*, multiple autoinducers are produced by autoinducer synthases and those autoinducers and corresponding sensor proteins act together to control the phosphorylation of the response regulator protein LuxO (Henke and Bassler, 2004). The phosphorylated form of LuxO allows for the production of multiple small RNAs (sRNAs), which repress the QS master regulatory protein LuxR (Figure 1.2). In a low cell density, sRNAs are activated from the phosphorylated LuxO and effectively repress the LuxR, while, in higher cell density, sRNAs are significantly

reduced thereby increasing the level of LuxR, resulting in the activation of the luminescence genes (Mok *et al*., 2003; Timmen *et al*., 2006; Waters and Bassler, 2005; Tu and Bassler, 2007).

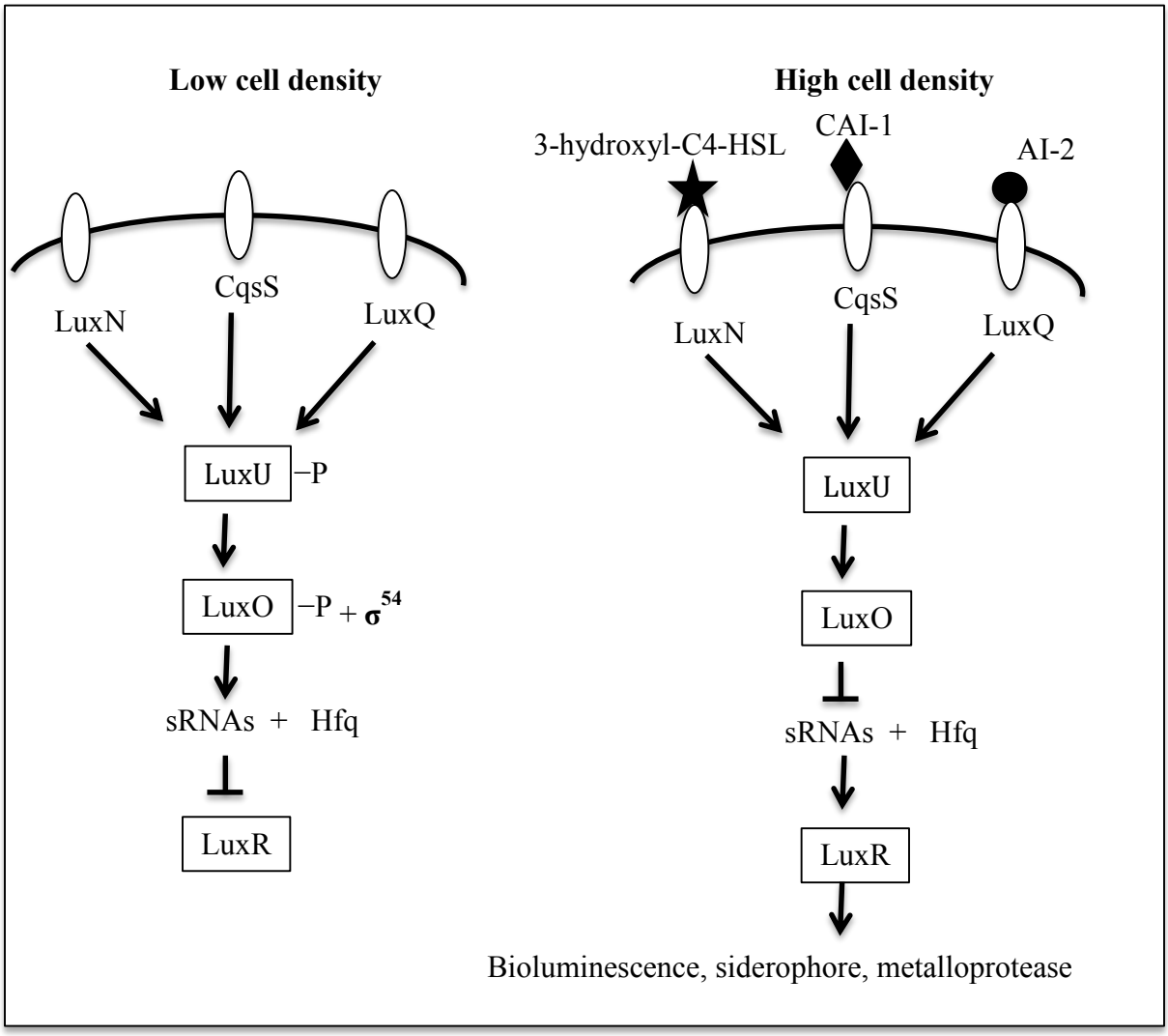

Figure 1.2. A schematic view of the quorum-sensing system of *V. harveyi.* In the low cell density, due to absence of autoinducers, the sensor kinases LuxN, LuxQ and CqsS autophosphorylate. Such a phosphorelay cascade phosphorylates LuxU and LuxO. Phosphorylated LuxO with sigma-54 activates the sRNAs. The sRNAs, along with Hfq, repress the *luxR* mRNA. In contrast, in high cell density, autoinducers interact with LuxN, LuxQ and CqsS sensors to switch from kinase to phosphatase. This results in the dephosphorylation of LuxO, making LuxO inactivate. The inactivate LuxO cannot promote the production of sRNAs, which allows the activation of *lux* operon by LuxR (McDougald *et al*., 2007).

2.2 Materials and Methods

2.2.1 Bacterial strains, media, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Burkholderia glumae* 336gr-1 and *Escherichia coli* were grown in Luria-Bertani (LB) broth or on LB agar plates (Sambrook *et al*., 2001) at 30˚C or 37˚C depending on the purpose of each experiment. Bacterial strains grown on LB broth were incubated in a shaking incubator at 200 rpm. In addition, recombinant mutants that lost the sucrose-sensitive gene, *sacB*, via the secondary homologous recombination were selected in LB agar plates containing 30% sucrose (Chen *et al*., 2012). Antibiotics used in this study were kanamycin (Km), nitrofurantoin (Nit), gentamycin (Gm) and ampicillin (Amp) at the concentrations of 50 µg/ml, 100 µg/ml, 20 µg/ml and 100 µg/ml, respectively.

Table 2.1. The bacterial strains and plasmids used in this study

Strains or plasmids	Properties	References
Escherichia coli S17-1λpir	*recA thi pro hsdR* [res-mod+][RP4::2-Tc::Mu-Km::Tn*7*] λ *pir* phage lysogen, Sm^r/Tp^r	(Simon *et al*., 1983)
Burkholderia glumae 336gr-1	WT strain and the causative isolate of bacterial panicle blight of rice in Crowley, LA	
Chromabacterium violaceum CV026	A biosensor that can detect AHL molecules	(McClean *et al*., 1997)
LSUPB401	A Δ*tepR* derivative of *B. glumae* 336gr-1	This study
LSUPB145	A Δ*tofI* derivative of *B. glumae* 336gr-1	(Chen *et al*., 2012)
pKKSacB	A suicide vector; R6K γ-*ori*, RP4 *oriT*, *sacB*, Km^R	(Chen *et al*., 2012)
pSC-A-amp/kan	A blunt PCR cloning vector; f1 *ori*, pUC *ori*, *lacZ*', Km^R, Amp^R	Stratagene
pBBR1MCS-5	A broad host range cloning vector, RK2 *ori*, *lacZα*, Gm^R	(Kovach et al., 1995)

(Table 2.1. continued)

Strains or plasmids	Properties	References
pRK2013::Tn7	A helper plasmid; ColE1 *ori*	(Ditta *et al*., 1980)

2.2.2 Development of a *tepR* deletion mutant

A markerless *tepR* (Locus_tag= "bglu_1g09700") deletion mutant (Δ*tepR*), LSUPB401, was developed using a pKNOCK vector derivative, pKKSacB, containing a kanamycin resistance gene and the *sacB* gene for sucrose sensitivity (Chen *et al*., 2012). Briefly, a 479-bp upstream flanking region of σ^{54} dependent response regulator gene (*tepR*) and a 355-bp downstream flanking region of *tepR* was amplified with the primers Sigma54GF2 (5'-CATGGTGCTGGTCTGCAA-3'), S54DWNR (5'-GTCGACGAAGACCTGTTGATCC-3'), S54UPF (5'-GTCGACGTTGGGATCGTCTT-3'), and S54UPR (5'-ATCGATAGATCACCTACAC-3'), respectively. The upstream and downstream PCR products were ligated separately into the pSC-A-amp/kan topocloning vector using the Strataclone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA). A *Sal*I-*Pst*I fragment containing the upstream region of *tepR* was then subcloned into pKKSacB to generate pKKSacBTepRUP. An *Xho*I-*Sal*I fragment containing the downstream region of *tepR* was subcloned into pKKSacBTepRUP to generate pKKSacBΔTepR. This work to make the construct (pKKSacBΔTepR) was done by research associate, Inderjit K. Barphaga in our laboratory for this study.

The pKKSacBΔTepR was first introduced into *E. coli* S17-1λ*pir* competent cells via electroporation and then introduced into *B. glumae* 336gr-1 via tri-parental mating with the helper strain pRK2013::Tn7. An initial selection following tri-parental mating was performed to obtain colonies resistant to kanamycin and nitrofurantoin. These colonies

were then grown at 30°C for 48 h on LB media with 30% sucrose. Colonies growing on the LB-sucrose plate were tested for their ability to grow on LB media without kanamycin and their inability to grow on LB media containing kanamycin. Colonies, which grew on LB media but not on LB media containing kanamycin were selected as markerless Δ*tepR* mutants. The deletion of *tepR* in those mutants was confirmed by PCR using primers TepRSuF (5'-GCTTGTTGTAGAGCGTCTTCG-3') and TepRSuR (5'-GGGGGACATAAAATCCGACT-3'), which amplify 1620 bp of *B. glumae* 336gr-1 and 219 bp for *tep*R deletion mutant. All the primers used for the experiments were listed in Table 2.2. For the confirmation of deletion following PCR condition was used:

1. Initialization step: 95°C for 5 min
2. Denaturation step: 95°C for 30 s
3. Annealing step: 55°C for 30 s
4. Extension/ elongation step: 72°C for 1.5 min
5. Go to step 2
6. Final elongation: 72°C for 7 min at last cycle (30th cycle)

Table 2.2. Primers and PCR conditions used in this study

Primer Name	**Primers (5'-3')**	**Annealing and extension condition**
S54UPF	GTCGACGTTGGGATCGTCTT	Annealing: 50°C/30 s
S54UPR	ATCGATAGATCACCTACAC	Extension: 72°C/50 s
S54GF2	CATGGTGCTGGTCTGCAA	Annealing: 50°C/30 s
S54DWNR	GTCGACGAAGACCTGTTGATCC	Extension: 72°C/30 s
TepRSuF	GCTTGTTGTAGAGCGTCTTCG	Annealing: 55°C/30 s
TepRSuR	GGGGGACATAAAATCCGACT	Extension: 72°C/1.5 min

2.2.3 Complementation

For the genetic confirmation of all the phenotypes showed by the Δ*tepR* mutant, complementation was done by cloning a functional DNA constructs carrying *tepR* into the Δ*tepR* mutant of *B. glumae* through triparental mating (Figurski and Helinski, 1979). The *tepR* clone for the complementation was developed by Mrs. Inderjit K. Barphaga, Department of Plant Pathology and Crop Physiology, LSU. For tripartental mating, overnight liquid culture of helper strain, Δ*tepR* and DNA construct containing functional *tepR* (pBB5-*tepR*, having Gm resistance marker) were used. From each of the overnight cultures, 500 µl was taken and mixed in a microcentrifuge tube and centrifuged in 1300 rpm for 1 min in centrifuge machine (Eppendrof Centrifuge 5415D). The resultant bacterial pellet was dissolved in 50 µl of LB broth and was dropped on an LB plate. The plate was incubated at 30°C overnight. On the next day, the spot on the plate was dissolved in 1 ml of LB broth and 100 µl of the sample was spread on LB Gm/ Nit plates. After incubation of those plates at 30°C for 2-3 days, colonies were observed. Colonies growing on the plates were again transferred to LB Gm/ Nit for confirmation. One of the several colonies that were confirmed to have the *tepR* clone by doing PCR was used for further study.

2.2.4 Toxoflavin quantification

Bacterial cells were grown on LB agar media at 37°C for 48 h. All bacterial cells were removed by sterile water from the surface of the agar plate and the concentration of the bacterial cell was measured through absorbance at 600 nm. The solid agar diffused with toxoflavin was chopped into small pieces. Then, the small pieces of the LB agar were mixed with chloroform 1:1 (w/v) ratio. After a few minutes, the chloroform fraction

that contains the toxoflavin was filtered through filter paper and collected in microtubes. The toxoflavin-dissolved chloroform was evaporated overnight in a fume hood. After evaporation, the remaining culture filtrate was dissolved in 1 ml of 80% methanol. For determining the relative amount of the toxoflavin, absorbance of each sample was measured at 393 nm (Jung *et al*., 2011) and 260 nm (Kim *et al*., 2004), using a spectrophotometer (Biomate 3, thermoelectromate corporation, USA).

Toxoflavin was also extracted from liquid culture of the strains. Bacterial cells were grown in LB broth for 48 h at 37˚C with shaking speed of 180 rpm. The concentration of bacteria was measured before proceeding for toxoflavin production. About 1 ml of the liquid culture ($OD_{600} = 1$) was centrifuged for 10 min at 10,000X g and cell free supernatant was collected in another centrifuged tube. The same volume (1 ml) of chloroform was added to the supernatant and the mixture was vortexed for 15 sec. The chloroform fraction was separated and evaporated in a fume hood overnight. After evaporation, the remaining culture filtrate was dissolved with 1 ml of 80% methanol. For determining the relative amount of the toxoflavin, the absorbance of each sample was measured at 260 nm (Kim *et al*., 2004).

2.2.5 Observation and quantification of lipase activity

Bacterial cells were grown in the LB broth overnight at 37˚C with shaking speed at 180 rpm. The lipase quantification was done according to Winkler and Stuckmann (1979) with some modifications as to obtain a cell-free supernatant, 1.5 ml of overnight culture was centrifuged for 10 min at 10,000 g. Substrate solution was made by adding 25 ml of isopropanol (Fischer Scientific, Fair Lawn, NJ, USA) containing 75 mg of *p*-nitrophenylpalmitate (Sigma-Aldrich, St. Louis, MO, USA), which was mixed with 90 ml

of 0.05M Sorensen's phosphate buffer (0.2 M NaH_2PO_4-5.3 ml and 0.2M Na_2HPO_4-94.7 ml, Sigma-Aldrich, St. Louis, MO, USA) with pH 8.0 containing 207 mg of Sodium deoxycholate (Acros Organic, NJ, USA) and 50 mg of gum Arabic (Acros Organic, NJ, USA). The freshly prepared substrate solution was then pre-warmed at 37^0C for 10 min and 2.4 ml of that solution was mixed with 0.1 ml of the cell free supernatant and again incubated in water bath at 37˚C for 15 min. After incubation the absorbance was measured on OD_{410} value in a spectrophotometer (Biomate 3, thermoelectromate corporation, USA).

2.2.6 Virulence test in onion bulb scales

The onion assay system used to determine the virulence of the bacterial strains in this study was similar to the system followed by Jacobs *et al*. (2008) for *Burkholderia cenocepacia* and Karki *et al.* (2012) for *B. glumae*. Fresh onion scales were cut into small pieces (~2*4 cm^2) with a sterile razorblade. A small puncture was made at the center of each onion piece with a sterile micropipette tip. An overnight liquid culture of bacteria grown at 37˚C with shaking speed at 180 rpm was centrifuged to obtain the pellet. The pellet was washed twice with LB broth and final bacterial suspension was made in 10 mM $MgCl_2$. The bacterial concentration was then adjusted to 5×10^7 CFU/ml (OD_{600} = 0.1). About 5 µl of the bacterial suspension was applied to the puncture of the onion scales. The inoculated onion scales were placed in moist chamber and incubated at 30˚C for 48-72 h. The virulence levels of each strains of *B. glumae* were evaluated by measuring the maceration area on each onion bulb scale.

2.2.7 Virulence test in rice

Rice (*Oryza sativa*) cultivar Trenasse was grown in the greenhouse in pots containing clay, commercial soil and sand in the ratio of 4:2:1 in the greenhouse. Overnight culture grown in LB plate at 37°C was resuspended in sterile water and the bacterial concentration was adjusted to 0.1 at OD_{600} (5×10^7 cfu/ml). The bacterial suspension was then sprayed onto the rice at the 20%-30% heading stage. For the proper disease development, the infected rice plants were covered with a plastic frame to maintain more than 80% humidity. The disease score was determined on the basis of observation of percentage-infected area of the panicles 7 days after the bacterial inoculation. The disease severity on rice panicles was determined by the following scale: healthy panicle, 0; 1%–10% symptomatic area, 1; 11%– 20% symptomatic area, 2; 21%–30% symptomatic area, 3; 31%–40% symptomatic area, 4; 41%–50% symptomatic area, 5; 51%–60% symptomatic area, 6; 61%–70% symptomatic area, 7; 71%–80% symptomatic area, 8; >81% symptomatic area, 9 (Nandakumar *et al*., 2007).

2.2.8 Hypersensitivity response test on tobacco leaves

For the hypersensitivity response test, tobacco plants were grown in greenhouse. Tobacco seeds were sterilized by rinsing with 100% isopropanol for 1 min. The seeds were again rinsed with 50% bleach and finally the seeds were rinsed seven times with sterile water. The surface sterilized tobacco seeds were germinated on MS media (MS salts-0.43 g, plant cell culture agar-0.75, sucrose-0.75 g, vitamins-10 µl, sterile water-100 ml) for 7 days. The germinated seeds were grown on plastic pots containing clay, commercial garden soil and sand in the ratio of 4:2:1 in the greenhouse. Overnight bacterial cultures grown on LB agar at 37°C were resuspended in 10 mM $MgCl_2$ and

adjusted to the OD_{600} value of 0.1 (5×10^7 cfu/ml). The resultant suspension was infiltrated in fully expanded tobacco leaves through the ventral side with the help of a needle-less syringe. About 10-12 week-old tobacco plants were used for this experiment. Hypersensitive response was observed after 18 h of infiltration.

2.2.9 Swimming and swarming test

To observe the motility action of the *B. glumae* strains, swimming and swarming tests were conducted on different agar concentrations. 0.3% LB agar and 0.7% LB agar medium were used for swimming and swarming tests, respectively following the procedure used by Kim *et al.* (2007). In this test about 1 ml of overnight culture was centrifuged at 13000 rpm for 1 min and washed with the LB broth twice. One µl (for swimming test) and 5 µl (for swarming test) of bacterial suspension were dropped on the center of freshly prepared LB agar plates. The inoculated plates were incubated at 37°C for 20 h.

2.2.10 Electron microscopy of flagella

Bacterial cells were grown in LB agar plates at 37°C and these cells were collected and suspended in sterile distilled water. Five µl of the bacterial cells in sterile water was spotted onto a square mesh copper grid. After 2 min, staining solution was added to the grid containing bacterial cells. After a minute, the liquid was withdrawn by using filter paper and morphology and presence or absence of flagella was observed using transmission electron microscopy (TEM) (Facility service provided by Socolofsky Microscopy Center, LSU).

2.2.11 AHL assay

Chromobacterium violaceum CV026 was used as a biosensor to determine the AHL production by *B. glumae* because in *C. violaceum* CV026 the purple pigment, violacein, can be restored by incubation with AHL signal molecules (McClean *et al*., 1997). The AHL production assay was performed following the procedure used by Kim *et al*. (2004). Briefly, the supernatant fraction of an overnight culture of each *B. glumae* strain grown in LB broth at 37°C was obtained by centrifugation. The AHL molecules were extracted from the supernatant (1ml) with an equal volume of ethyl acetate. The ethyl acetate fraction, which dissolved AHL molecules, was left in a fume hood for air-drying. The residue obtained from the air-dried sample was dissolved in 1% volume of sterile distilled deionized water. Then, 20 µl of each culture extract were applied to the cells of *C. violaceum* CV026 immediately after they were streaked on a LB agar plates. The production of the purple pigment by this biosensor strain was observed after 48 h of incubation at 30°C.

2.2.12 Protease activity test

Protease activity was compared among the bacterial strains by spotting 5 µl of each bacterial suspension with OD_{600} = 1.0 onto the surface of nutrient yeast glycerol agar (NYGA) plate containing 1% skimmed milk powder (Huber *et al*., 2001). The plates were incubated at 37°C and observed at 24 h and 48 h of incubation.

2.3 Results

2.3.1 Development of markerless deletion mutant of *tepR* and complemented strain

A mutant derivative of *B. glumae* 336gr-1 with deleted *tepR* region, LSUPB401, was generated using the pKKSacB system (Chen *et al*., 2012). The *tepR* deletion in

LSUPB401 was confirmed by PCR using primers corresponding to the flanking sequences of the deleted region (Figure 2.1 and Table 2.2). A functional *tepR* clone, pBB5-*tepR* was introduced into the *tepR* deletion mutant, LSUPB401, through triparental mating. The sizes of the PCR products amplified from the wild type and the mutant matched the predicted sizes of the intact and deleted *tepR* DNA sequences (Figure 2.1). The complemented strain, LSUPB401 (pBB5-*tepR*), was confirmed by the amplification of both intact and deleted *tepR* regions (Figure 2.1).

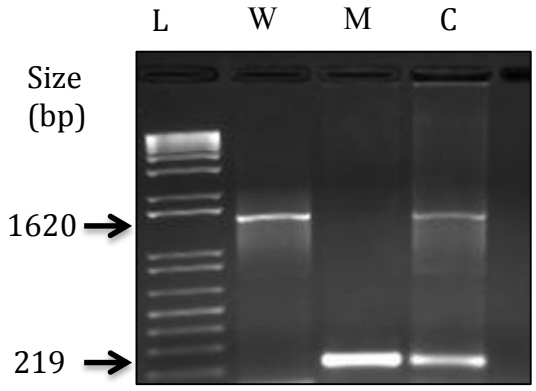

Figure 2.1. PCR products amplified with the primers, TepRSuF and TepRSuR, to confirm the *tepR* deletion in LSUPB401. L indicates the 1kb plus DNA ladder (Invitrogen, Santa Clara, CA, USA) used as a marker; W indicates the wild type strain, 336gr-1; M indicates the *tepR* deletion mutant, LSUPB401; and C indicates the complemented strain, LSUPB401(pBB5-*tepR*).

2.3.2 Toxoflavin production assay

The *tepR* deletion mutant of *B. glumae* 336gr-1, LSUPB 401, produced higher toxoflavin production compared with its parental strain when grown in LB agar plate (Figure 2.2). Similar results were observed when the bacterial strains were grown in LB broth (Figure 2.3). Statistical analysis showed significantly higher toxoflavin production by *tepR* deletion mutant in liquid media (Figure 2.4).

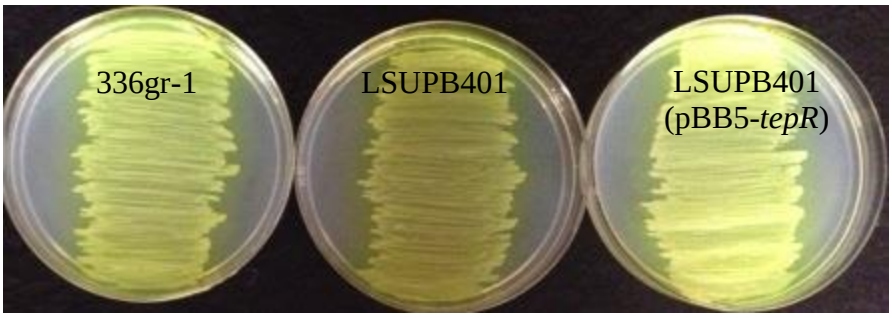

Figure 2.2. Toxoflavin production by *B. glumae* strains in LB agar plates. The *tepR* deletion mutant LSUPB401 showed enhanced toxoflavin production compared with the parental wild type strain, 336gr-1, when growing on LB agar for 48 h at 37°C.

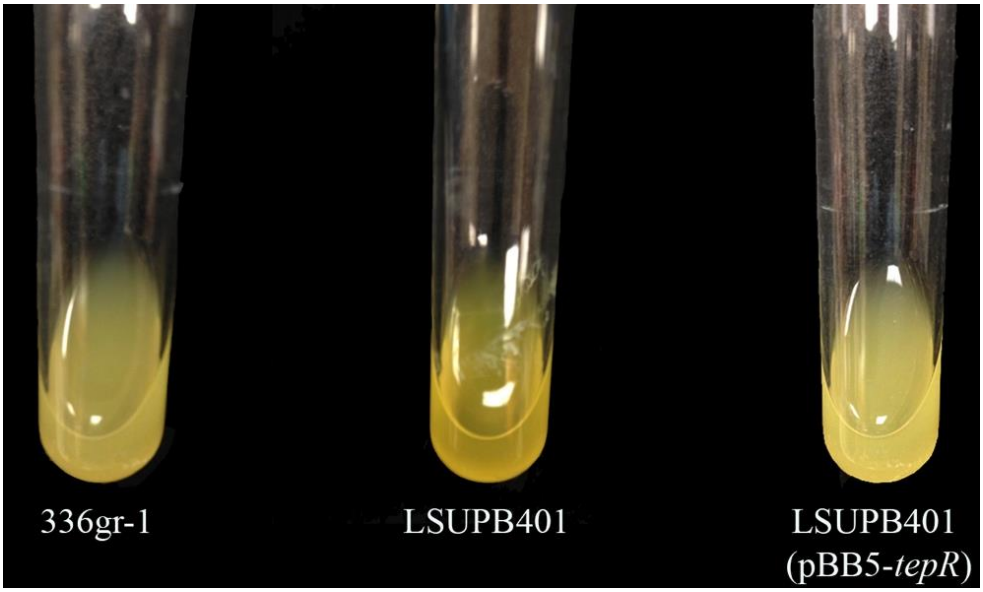

Figure 2.3. Toxoflavin production by *B. glumae* strains in LB broth. The *tepR* deletion mutant, LSUPB401, yielded higher toxoflavin in comparison with the parental wild type strain, 336gr-1, when growing in LB broth for 48 h at 37°C.

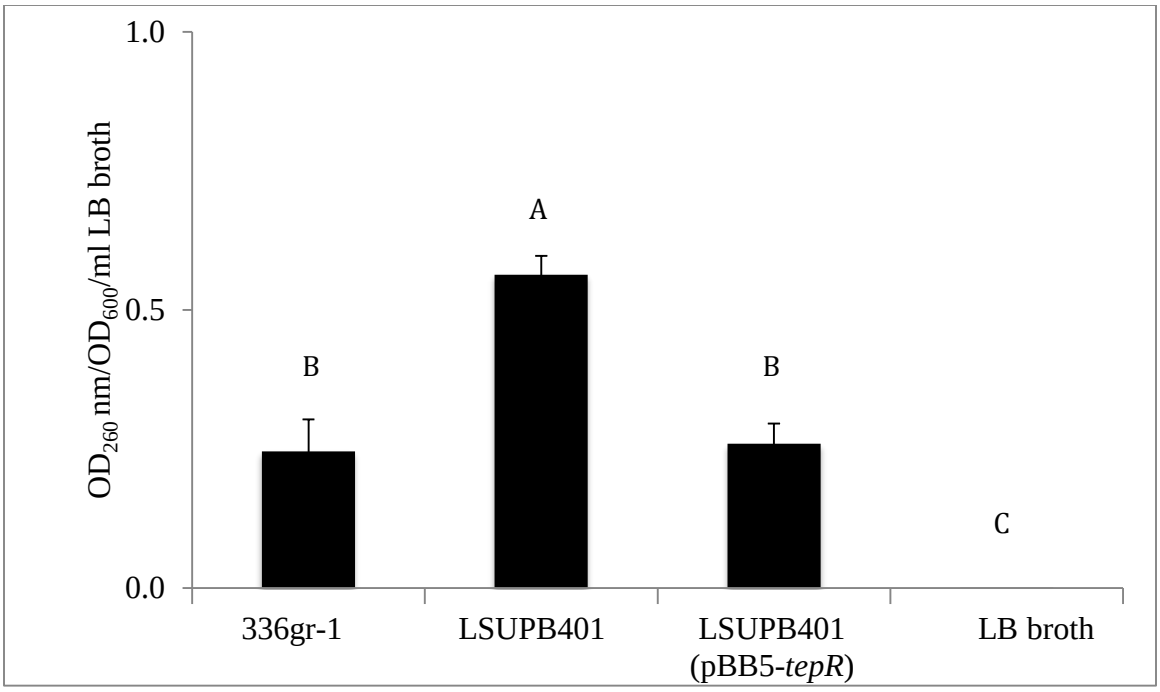

Figure 2.4. The *tepR* deletion mutant of *B. glumae*, LSUPB401, produced significantly higher amount of toxoflavin than its parental wild type strain, 336gr-1. Toxoflavin was quantified measuring the toxoflavin dissolved in 80% methanol at an optical density 600 nm (OD_{610}). All experiments were done more than three times. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with a same letter are not significantly different.

According to the statistical analysis using Tukey's honest significant difference (HSD) test at P value 0.005 (Tukey, 1975), toxoflavin production in liquid LB media was significantly different between the wild strain and the *tepR* deletion mutant, LSUPB401 (Figure 2.4).

2.3.3 Lipase activity

The Δ*tepR* strain, LSUPB401, showed higher lipase activity compared to its wild type strain 336gr-1. The mean of the OD_{410} values, which represented the relative amount of the *p*-nitrophenol formed after the reaction of lipase on the substrate (*p*-nitrophenyl palmitate), was plotted on a bar chart (Figure 2.5). The bar chart showed that Δ*tepR* had relatively higher lipase activity than that of the wild type. The statistical analysis by

using Tukey's honest significant difference (HSD) test at P value 0.05 (Tukey, 1975), suggested the significantly higher lipase activity in LSUPB401 compared to wild type strain 336gr-1 (Figure 2.5).

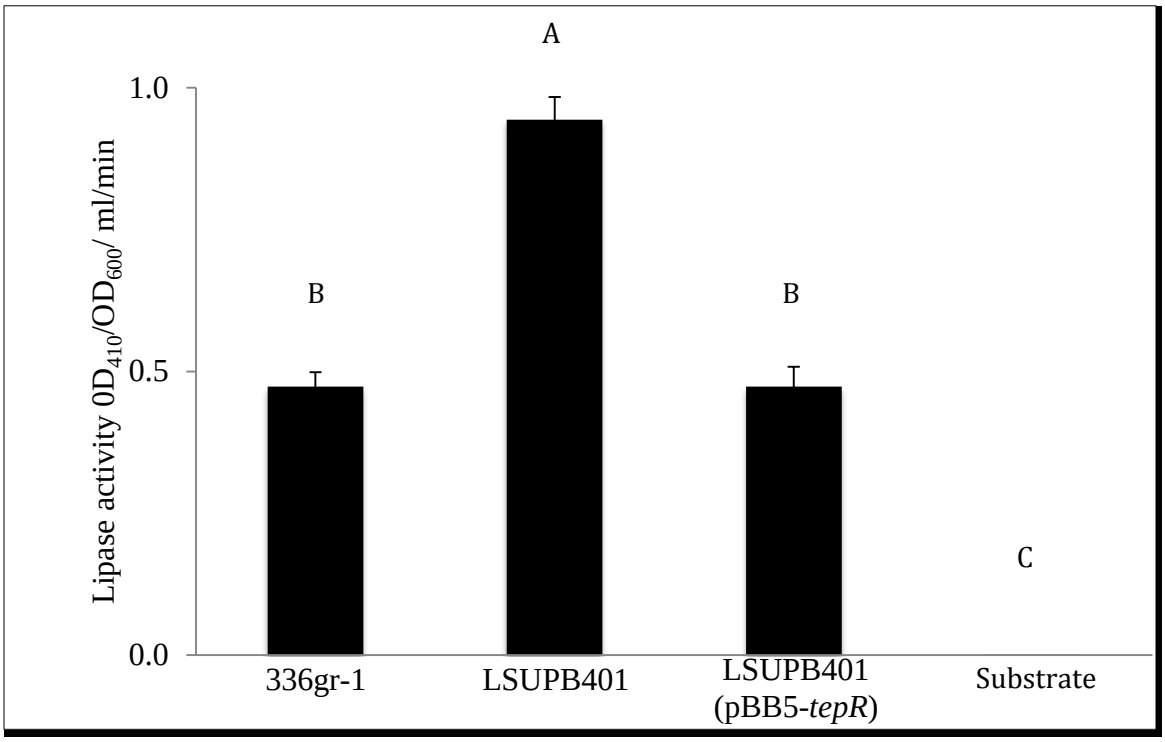

Figure 2.5. A *tepR* deletion mutant of *B. glumae,* LSUPB401, showed higher lipase activity than the parental wild type strain, 336gr-1. Lipase activity was quantified measuring the release of *p*-nitrophenol from the chromogenic substrate *p*-nitrophenyl palmitate at an optical density at 410 nm (OD_{410}). All experiments were done in triplicate. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with a same letter are not significantly different.

2.3.4 Virulence assay on onion

In the onion bulb scale assays, *ΔtepR* exhibited a wider maceration area than the wild type strain, 336gr-1(Figure 2.6). The size of maceration area caused by a *B. glumae* strain is thought to reflect its virulence activity. The maceration area on an onion bulb scale was in elliptical shape, so the size of each maceration area was measured by using the formula $A= \pi (R1*R2)$; where R1 and R2 are radius of ellipse in horizontal and vertical directions, respectively, and A is the maceration area.

The statistical analysis suggested that LSUPB401 macerated the onion scale significantly better than its parental strain, 336gr-1 (Figure 2.7) on the moisture chamber at 30°C when observed after 48 h.

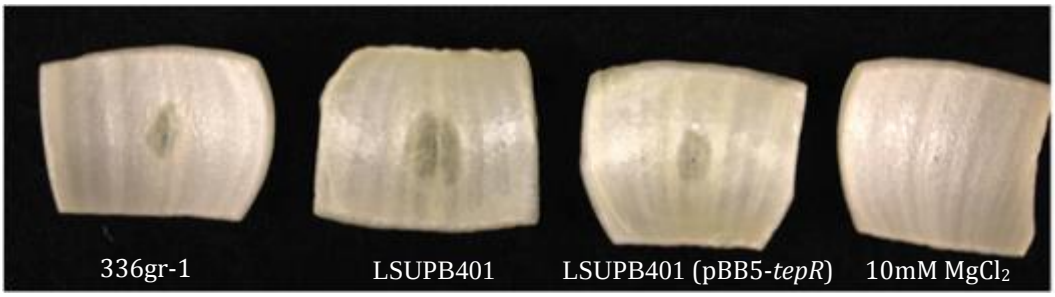

Figure 2.6. Maceration on onion scales caused by different strains of *B. glumae* 336gr-1. A *tepR* deletion mutant of *B. glumae,* LSUPB401, showed a larger maceration area than its parental wild type strain, 336gr-1, on onion scales.

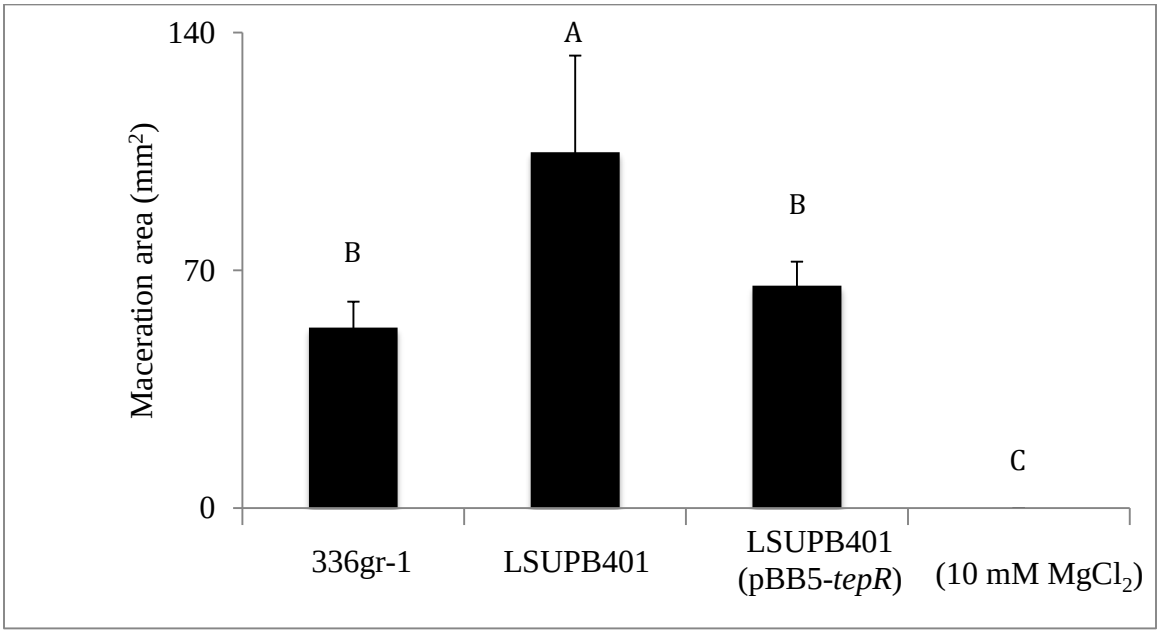

Figure 2.7. A *tepR* deletion mutant of *B. glumae,* LSUPB401, showed larger maceration areas on onion scales. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with the same letter are not significantly different.

2.3.5 Virulence assay on rice

In the rice virulence test performed in the greenhouse, LSUPB401 showed more severe disease symptoms than the wild type, 336gr-1 (Figure 2.8). Statistical analysis of the disease scores confirmed the significantly higher virulence of LSUPB401 compared with the wild type and complemented strains (Figure 2.9).

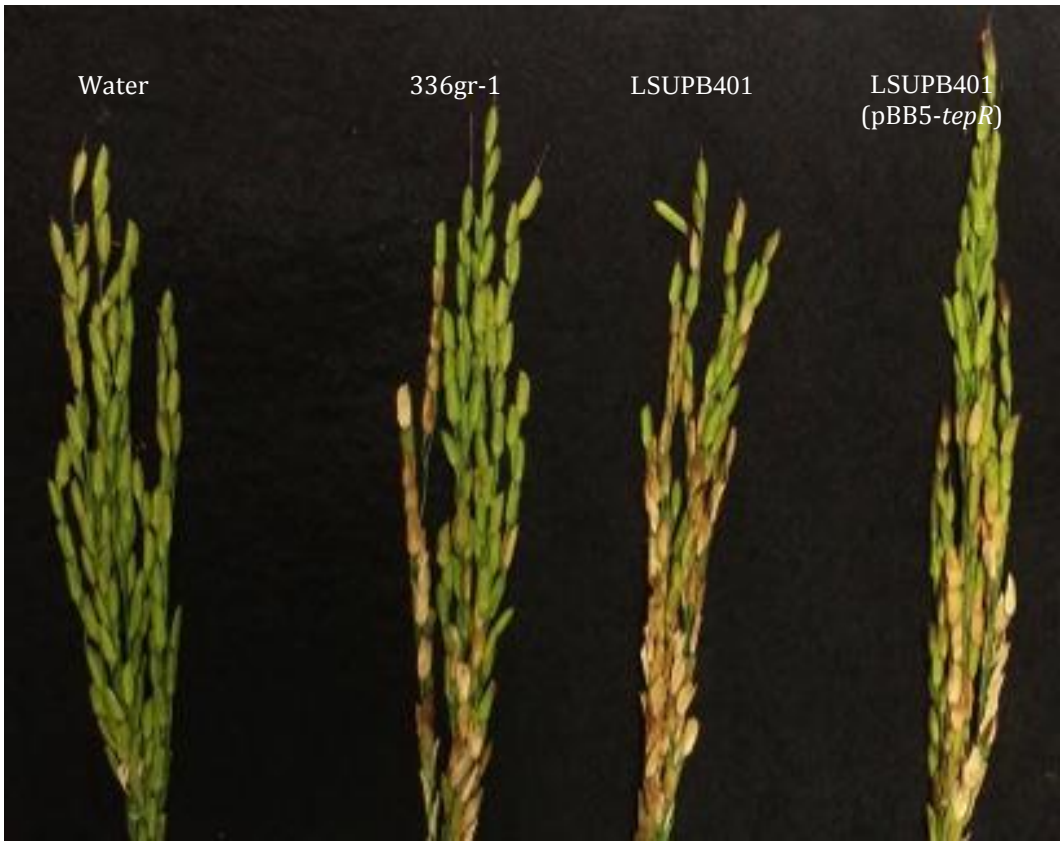

Figure 2.8. Disease symptoms on rice panicles caused by different *B. glumae* strains in the greenhouse. LSUPB401 is more aggressive than the parental strain, 336gr-1, in causing bacterial panicle blight symptoms.

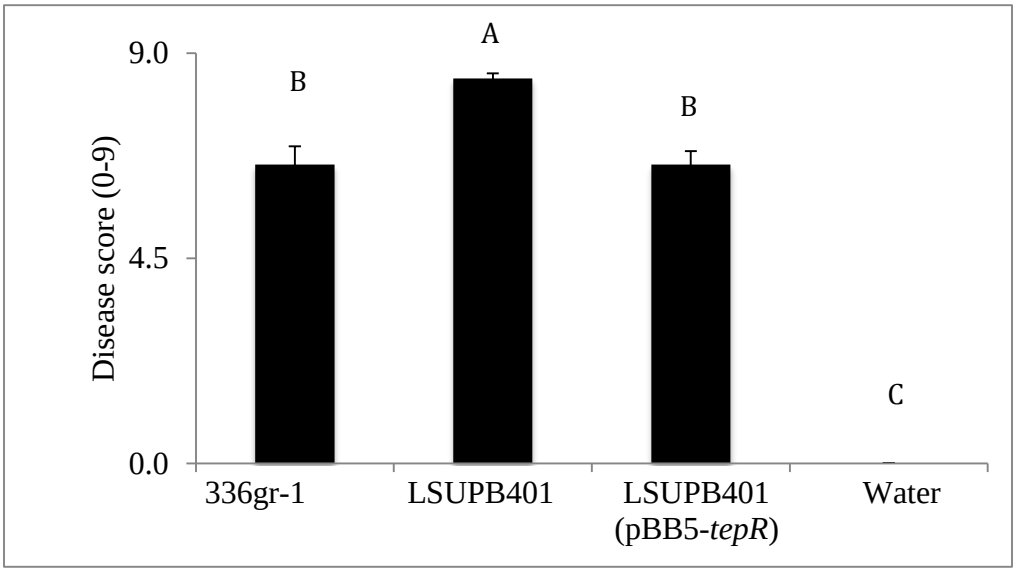

Figure 2.9. A *tepR* deletion mutant of *B. glumae,* LSUPB401, showed more severe disease symptoms in rice panicles compared with the wild type and complemented strains. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with a same letter are not significantly different.

2.3.6 Hypersensitive responses (HRs) in tobacco leaves

Unlike the wild type 336gr-1, its *ΔtepR* derivative, LSUPB401, did not elicit any HR in tobacco leaves when the bacterial cells were infiltrated in a fully expanded tobacco leaf in the greenhouse condition (Figure 2.10).

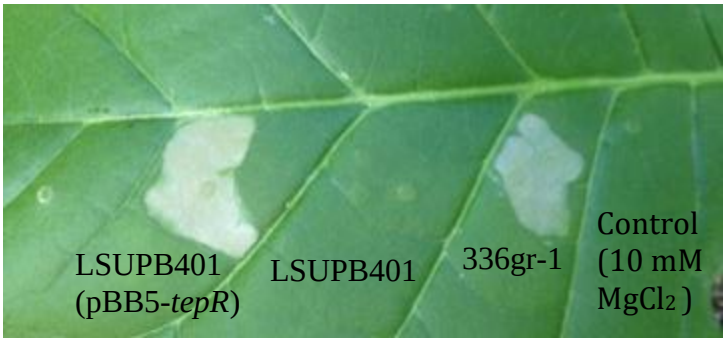

Figure 2.10. Hypersensitivity response (HR) in tobacco leaf, where the *ΔtepR* strain, LSUPB401, does not elicit HR.

2.3.7 Protease activity

Protease is also known to be an important factor of plant pathogens to cause disease. LSUPB401 showed higher protease activity in comparison to its wild type parent on the surface of nutrient yeast glycerol agar (NYGA) plate containing 1% skimmed milk powder (Figure 2.11).

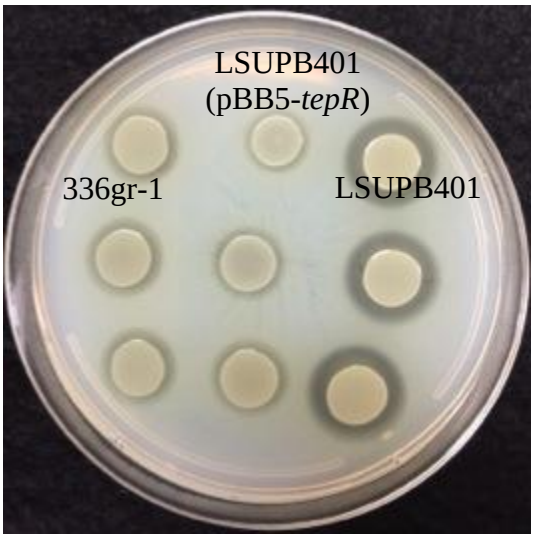

Figure 2.11. Protease activities of *B. glumae* strains on NYGA containing 1% skimmed milk. Photo was taken after 24 h of incubation at 37°C.

2.3.8 AHL signal production assay

In AHL assays using the biosensor, *Chromobacterium violaceum* CV026, LSUPB401 (*ΔtepR*) was negative in AHL production, while the culture extract of the wild strain, 336gr-1, caused the production of the purple pigment (violacein) by the biosensor indicating the presence of AHL (Figure 2.12).

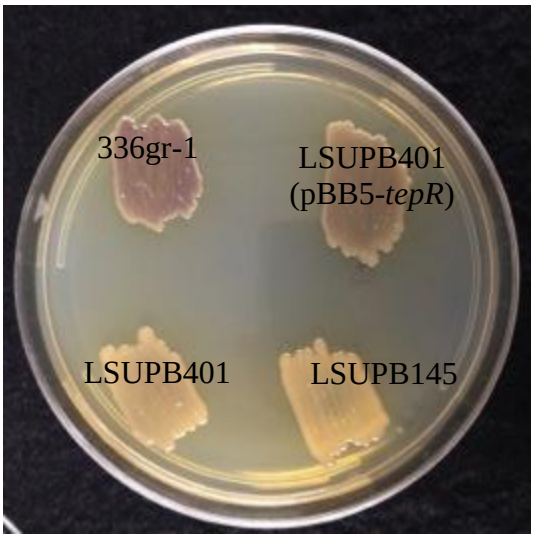

Figure 2.12. Production of AHL compounds by *B. glumae* strains. AHL production is indicated by the purple pigment produced by the biosensor, *Chromobacterium violaceum* CV026. 336gr-1: wild type. LSUPB401: *ΔtepR*. LSUPB145: *ΔtofI*. Photo was taken after 48 h of incubation at 30°C.

2.3.9 Swimming and swarming activity

In LB agar media with 0.3% agar, *ΔtepR* showed no swimming activity (Figure 2.13). Similarly, *ΔtepR* did not show any swarming activity on LB with 0.7% agar (Figure 2.14). However, wild strain, 336gr-1 showed both swimming and swarming activities.

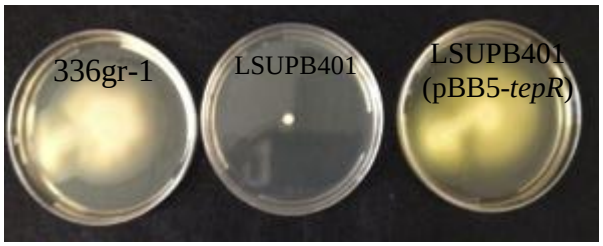

Figure 2.13. Swimming activities on 0.3% LB agar plates at 37°C. Photo was taken after 20 h of incubation at 37°C.

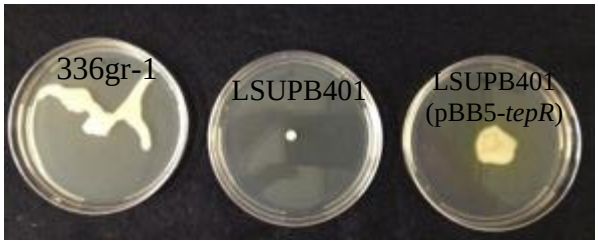

Figure 2.14. Swarming activities on 0.7% LB agar plates at 37°C. Photo was taken after 20 h of incubation at 37°C.

2.3.10 Electron microscopy of bacterial cells for the observation of flagella

Electron microscopy observation of showed that *ΔtepR* did not show flagella, where as the wild type and the complemented strain of the *tepR* deletion mutant showed distinguishable flagella (Figure 2.15).

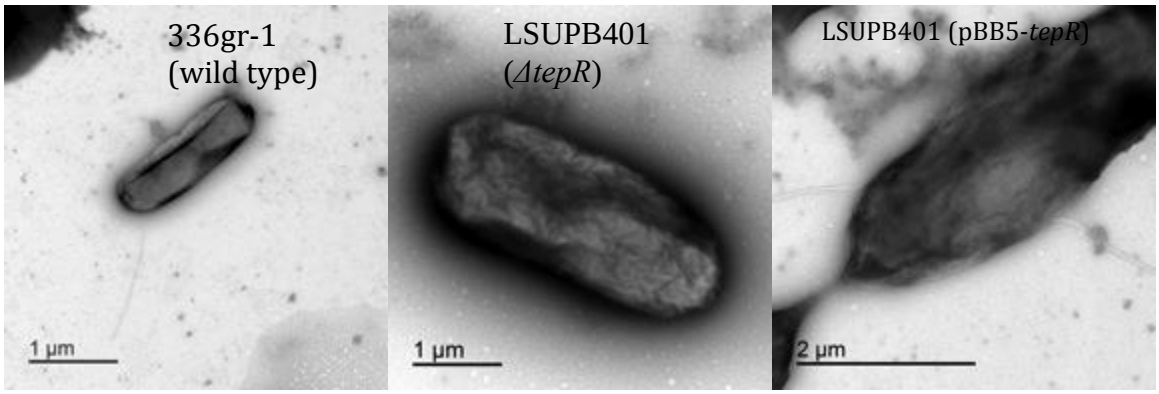

Figure 2.15. Flagella observed under electron microscope. The wild type strain, 336gr-1 (left), and LSUPB401 (pBB5-*tepR*) (right) show flagella formation but the *tepR* deletion mutant, LSUPB401 (center), did not.

2.4 Discussion

B. glumae is one of the major bacterial pathogens of rice plant, which causes bacterial panicle blight and reduces the yield up to 75 % in severe infestation. *B. glumae* has several known virulence factors. Major known virulence factors of the bacteria are toxoflavin, lipase and flagellar motility. The major objective of this study was to characterize the σ54-dependent response regulator, *tepR* (Locus_tag= "bglu_1g09700"),

which was assumed to be a negative regulator for the toxoflavin production in *B. glumae*. In a genome-wide search of regulatory factors by using of transposons (Tn5) for random mutagenesis, some mutants showed higher toxoflavin production and some showed no toxoflavin. One of the mutants showing higher toxoflavin production was further analyzed and found that the transposon was inserted on a putative gene encoding a σ^{54}-dependent response regulator. The gene where the transposon was inserted was named *tepR* (**t**oxoflavin and **e**xtracellular **p**olysaccharides **r**egulotor). Those tasks were already completed in our laboratory before this study.

TepR (protein id= "ACR28150.1") of *B. glumae* was found to be homologous with the LuxO of *Vibrio* spp. It was already known that in *V. harveyi*, *luxO*, along with sigma-54, acts as a negative regulator for the luminescence (Lilley and Bassler, 2000). Based on the known *luxO* function, the *luxO* homolog *tepR* was hypothesized to be a negative regulator and studied further by creating a markerless *tepR* deletion mutant, LSUPB401. The *ΔtepR* strain, LSUPB401, along with its wild type parental strain, 336gr-1, and the complemented strain with a functional *tepR* clone, LSUPB401 (pBB5-*tepR*), was further characterized in terms of various phenotypes.

Toxins are major components of plant pathogenic bacteria to cause disease in plants (Durbin, 1991) through either injuring the plant organelles or disrupting normal metabolic processes of the host. Toxoflavin is an important toxin produced by *B. glumae* and considered a crucial virulence factor for the symptoms development in plant by producing superoxide and hydrogen peroxide (Latuasan and Berends, 1961; Jung *et al*., 2011). In this study the *tepR* deletion mutant of *B. glumae* showed higher toxoflavin production than the wild type in both liquid and solid medium conditions. In both

conditions, LSUPB401 complemented with a functional *tepR* clone showed the similar amount of the toxoflavin production to the wild type. Those results suggested that *tepR* negatively regulates the toxoflavin production in *B. glumae*.

Lipase is another important microbial enzyme, which is involved in virulence activity of various plant pathogens including fungi and bacteria. Lipase was found to be involved actively in the pathogenicity of the rice blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Rajeshwori *et al*., 2005). According to Devescovi *et al*., (2007) *lipA* mutants of *B. glumae* AU6208 showed almost no disease symptoms in rice, suggesting an important role of lipase in the virulence of *B. glumae*. In this study, the higher lipase activity shown by LSUPB401 indicated the negative regulatory role of *tepR* in lipase activity. Devescovi *et al*., (2007) described that lipase production in *B. glumae* is regulated by the AHL- dependent quorum sensing system. However, LSUPB401 did not show AHL production even though it showed higher lipase activity. In some species of *Burkholderia*, such as *B. thailandensis*, lipase production was controlled by the AHL-dependent quorum sensing in both negative and positive ways (Ulrich *et al*., 2004). So, it is interesting that *tepR* represses toxoflavin production and lipase activity even if it positively regulates the AHL production. Studying how toxoflavin production and lipase activity of *B. glumae* increase in the *ΔtepR* background without AHL would be an important part of the future study on TepR function. Regarding this question, it is noteworthy that *ΔtofI* and *ΔtofR* strains of *B. glumae* did not produce AHL signals but they were able to produce high levels of toxoflavin when grown on LB agar (Chen *et al*., 2012).

Interestingly, the *tepR* deletion mutant showed a higher protease activity on the surface of NGYA plates containing 1% skimmed milk, suggesting a negative regulatory role of *tepR* in the protease activity of *B. glumae*. This result was similar to the result obtained by Raychaudhuri *et al*., (2006) with *V. cholera* strain PL91, where deletion of *luxO* enhanced the protease activity than the wild type and a constitutively active LuxO caused protease-deficient phenotype. Taken together, the obtained results indicated that *tepR* negatively regulates some important virulence factors of *B. glumae*.

The HR test in this study indicates that the *tepR* positively regulate the type III secretion system of *B. glumae*. Motility by flagella is an important virulence activity of bacteria. Flagella are complex molecular machines, which allow the cell movement (McCarter, 1988). Flagella are essential parts developed by the pathogenic bacteria for the movement and attachment on host tissue during the establishment phase of the infection (Davey and O'toole, 2000). A single polar flagellum is involved in swimming activity in less viscous medium, whereas numerous lateral flagella are responsible for the swarming over the more viscous surface (Yang and Defoirdt, 2014). It was very interesting that LSUPB401 lost the flagellum-mediated swimming and swarming activities at 37°C. This result was further validated by electron microscopy observations, in which the *tepR* deletion mutant did not show flagella but its complemented strain having the functional *tepR* clone retained flagella. Those results suggest that *tepR* plays a positive role in flagellar biogenesis and flagellum-mediated motility even if it negatively regulates other major virulence factors including toxoflavin and lipase. Furthermore, LSUPB401 was more virulent on rice and showed higher disease severity than its wild type strain. Similar results were obtained in onion virulence assays, in that the *tepR*

deletion mutant showed larger maceration areas on onion bulb scales than the wild type strain.

Virulence of *B. glumae* in relation to the toxoflavin production, lipase activity and flagella-dependent motility is an important part of the *B. glumae* study. Kim *et al*, 2007 observed that a non-motile mutant of *B. glumae* produced toxoflavin but failed to cause disease in rice. This study reveals a very interesting fact that deletion of *tepR* caused increased virulence of the pathogen in spite of the lost flagellum-mediated motility and the deficiency in AHL production. It might be possible that *tepR* regulates *B. glumae* positively for the initial colonization on the host tissue but does negatively for the production of major virulence factors at later infection stages. This speculation may be reasonable because bacteria need to manage proper amounts of virulence factors after being established in host for long-term survival.

The newly found functions of the *luxO*-type regulatory gene *tepR* would provide insights into the regulatory/signaling network of *B. glumae* and related bacteria, and this study would be the foundation for further mechanistic studies of the *tepR* function.

CHAPTER 3. ISOLATION OF RICE RHIZOSPHERIC BACTERIA TO FIND OUT POTENTIAL BIO-CONTROL AGENTS FOR BACTERIAL PANICLE BLIGHT IN RICE

3.1 Introduction and literature review

Rice is one of the most important staple food crop in the world because it is a source of abundant carbohydrates and more than 3.5 billion people are dependent on it for their daily energy consumption (Spence *et al*., 2014). Rice diseases are major limiting factors in the productivity of rice. Bacterial panicle blight is an important bacterial disease of rice and no effective management system has been established to control this disease.

Pathogenic microorganisms affecting plant health and surrounding environment are major threats for agriculture and food science. To maintain the intense agricultural production, producers use different agrochemicals for crop protection (Compant *et al*., 2005). However usage of agricultural chemicals may not be good for the environment. The possibility of developing resistance by plant pathogens to the applied chemicals in the field and non- target impacts of those chemicals in an environment suggest the need of an alternative way for plant disease management. Endophytic bacteria are used as important biocontrol agents used against different pathogenic bacteria of crops (Jacobsen *et al*., 2004). Nowadays, biological control is considered as an alternative way of reducing the chemical usage and minimizing environmental pollution (De Weiger *et al.,* 1995). Free-living bacteria and some endophytic bacteria use different mechanisms to promote the plant growth and to control plant pathogens (Glick, 1995). Antibiosis, lytic enzyme production, detoxification and degradation of virulence factors are some important bases of biocontrol mechanism of plant-associated bacteria and these mechanisms are increasingly studied over the past decades (Compant *et. al*., 2005). A

plant growth-promoting bacterium *Delftia tsuruhatensis*, strain HR4, which was isolated from rhizosphere of rice in North China, showed a suppression activity on the growth of major rice pathogens; *Xanthomonas oryzae* pv. *oryzae*, *Rhizoctonia solani* and *Pyricularia oryzae* Cavara on *in vitro* antagonistic assay (Han *et al*., 2005). Ji *et al*., (2008) isolated a novel strain of *Lysobacter antibioticus* (named as *L. antibioticus* strain 13-1) from the rhizosphere of rice in Yunan Province of China and the isolated bacteria inhibited growth of the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae*, suggesting its value as a potential biocontrol agent.

In nature many bacteria produce different antibiotics along with some other chemical compounds. Along with antibiotics, pseudomonads produce a variety of chemical compounds such as amphisin, 2,4- diacetylphloroglucinol, hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone and cyclic lipopeptides (Défago, 1993; de Souza *et al*. 2003; Nielsen *et al*. 2002, Nielsen and Sørensen, 2002). In a similar way, oligomycin A, kanosamine, zwittermicin A and xanthobaccin are produced by *Bacillus, Streptomyces* and *Stenotrophomonas* spp. (Hashidoko *et al*., 1999; Kim *et al*., 1999; Milner *et al*., 1995; Milner *et al.*, 1996; Nakayama *et al.*, 1999). Detoxification of bacterial virulence factors is another way to control pathogenic bacterium. During the detoxification, certain proteins produced by biocontrol agents binds to the toxin of pathogenic bacterium to deactivate its toxicity. Esterase of *Pantoea dispersa* detoxifies the albicidin toxin produced by *Xanthomonas albilineans* (Zhang and Birch, 1996, 1997). Moreover, some bacteria can control fungal toxins too. For example, *Burkholderia cepacia* and *Ralstonia solanacearum* can hydrolyze the fusaric acid of *Fusarium* spp. (Toyoda and Utsumi, 1991). It was also reported that some plant growth promoting

bacteria disturb the quorum sensing signals by degrading auto-inducers (Dong and Zhang, 2005).

The rhizosphere is a small and narrow zone of soil around the root system of plants, which is very rich in nutrients due to the accumulation of different plant exudates like amino acids and sugars. These are an important source of energy and nutrients for diverse microorganisms including bacteria (Gray and Smith, 2005). Bacteria residing in the rhizosphere are called rhizobacteria and they can be differentiated into beneficial, deleterious and neutral groups based on their effects on plants (Dobbelaere *et al*., 2003). The interaction between plant roots and their rhizospheric microbiome are crucial to plant fitness and important driving forces for both growth promotion and disease suppression (Spence *et al*., 2014). Rhizospheric bacteria are potential biological control agents against different plant diseases and also efficient promoters of plant growth, therefore providing additional benefits (Narayanasamy, 2013).

Rhizospheric microbial communities not only get benefit from the plants exudates but also aid in nutrient accumulation in the plants, control different soil pathogens by producing antimicrobial compounds, and through the competition with pathogens for nutrients (Lugtenberg and Kamilova, 2009). In most cases, gram-positive bacteria within the genus *Bacillus* are used as biocontrol agents and studies have shown that these bacteria produce surfactins with antimicrobial activity (Vitullo *et al*., 2012). Similarly, some well-studied gram-negative bacteria as biocontrol agents, such as *Pseudomonas* species, have also been shown to secrete a large number of antimicrobial metabolites (Silby *et al*., 2011). It is known that certain plant-associated strains of fluorescent *Pseudomonas* spp. produce the antimicrobial antibiotic 2,4-diacetylphloroglucinol

(DAPG), which has antibacterial, antifungal, antiviral and antihelminthic properties. Velusamy *et al*., (2006) reported that DAPG inhibited the growth of rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in laboratory assays and also suppressed the bacterial blight up to 60% in greenhouse and field experiments.

Host immunity and defense responses are important governing factors in a host pathogen interaction. Through the alteration of defense responses of host, different biocontrol agents act as a priming factor on host to recognize pathogens earlier and ultimately make hosts immune against the pathogen (Van Loon, 2007). For the induced systemic resistance (ISR) response of plants, jasmonic acid (JA) and ethylene (ET)-mediated plant signaling systems are active in most cases. Microbial cell components, volatile substances and secretions are recognized by the plants as priming factors for the rapid cellular defense responses (Van der Ent *et al*., 2009: Ryu *et al*., 2004: Van Wees *et al*., 2008).

The objective of this study was to isolate and characterize rhizospheric rice bacteria in order to identify potential bio-control agents for controlling bacterial panicle blight.

3.2 Materials and Methods

3.2.1 Isolation of bacteria from rice rhizosphere

Rice plants with root and soil adhering to the root surface were collected from the rice fields at the Rice Research Station (Crowley, LA). The soil adhering the root surface was used to isolate rhizospheric bacteria. Each soil sample was suspended in double distilled water and filtered through a filter paper. Then the filtrate was diluted to 1/10 and 1/100 and spread (100 µl) on Nutrient agar (NA) plates containing cyclohexamide (50

µg/ ml). The plates were incubated at 30°C overnight. Bacterial colonies growing on the plates were picked with sterile toothpicks and transferred to another nutrient agar plate where *Burkholderia glumae* (OD_{600} = 0.1) was spread. Those plates were further incubated at 30°C overnight. The bacterial colonies showing the antagonistic characteristics (inhibition zones) against *B. glumae* were used for further studies.

3.2.2 *In vitro* screening

The bacterial isolates showing inhibition regions on *B. glumae* plate were tested further to check antagonistic characteristic against the *B. glumae.* For this experiment a bacterial suspension of the *Burkholderia glumae* 336gr-1(OD_{600} = 0.1) was spread on nutrient agar plates. One ml culture of each selected rice rhizospheric bacteria grown overnight in LB broth at 37°C was centrifuged to obtain a pelleted bacterial cells. The cells were washed with equal volume of fresh LB broth two times and re-suspended in 100 µl LB broth. Ten µl of each bacterial sample was spotted in the center of a plate spread with *B. glumae*. Those plates were incubated overnight at 30°C. Next day, the areas of inhibition region were measured and analyzed using Tukey's honest significance difference (HSD) test with *P* value, 0.05 (Tukey, 1975).

3.2.3 Characterization of the bacteria

The isolated bacteria were differentiated into gram positive and gram negative on the basis of KOH test (Powers, 1995). For this test 3% KOH solution was prepared and a drop (50 µl) of KOH was put on clean glass slide. The bacterial cells grown overnight on nutrient agar plates were transferred to the slide aseptically with a flat toothpick into the drop of KOH solution and resuspended by rapid and circular agitation. After 5-8 seconds, the toothpick was alternately raised and lowered from the slide surface. The observation

was made to determine whether viscous and mucoid string is formed or not.

Those bacteria showing the antagonistic characteristics against *B. glumae* were proceed for genomic DNA extraction. The 16S-rDNA region of each bacterium was amplified by using universal primers (the forward primer 5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG 3' and the reverse primer 5'CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC 3') (Weisburg *et al.,* 1990). The amplified DNA samples were sent to a genome service facility for DNA sequencing. The sequence result was evaluated by using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

3.2.4 Greenhouse study

The antagonistic bacteria were tested against bacterial panicle blight in the rice plants (Trennase cultivar) grown in the greenhouse in pots containing clay, commercial soil and sand in the ratio of 4:2:1 in the greenhouse. Bacterial suspensions of antagonistic bacteria were prepared from the overnight grown bacterial culture in NA plates with the OD_{600} value 0.1 and sprayed on the rice plants at the 20-30% heading stage. On the following day, *B. glumae* suspension (OD600=0.1) was sprayed on the pretreated rice plants. More than 85% relative humidity and around 37°C temperature were maintained inside the greenhouse to develop the bacterial panicle blight disease on the rice plants. Disease scoring was done one week after inoculation of isolated bacteria. The resulting disease ratings were evaluated by using Tukey's honest significance difference (HSD) test with *P* value, 0.05 (Tukey, 1975). The disease severity on rice panicles was determined by the following scale: healthy panicle, 0; 1%–10% symptomatic area, 1; 11%– 20% symptomatic area, 2; 21%–30% symptomatic area, 3; 31%–40% symptomatic

area, 4; 41%–50% symptomatic area, 5; 51%–60% symptomatic area, 6; 61%–70% symptomatic area, 7; 71%–80% symptomatic area, 8; >81% symptomatic area, 9 (Nandakumar *et al*., 2007).

3.2.5 Field test

Bacterial suspensions of antagonistic bacteria were prepared with the OD_{600} value 0.1 and sprayed on the rice plants at the 20-30% heading stage, which were grown in the Rice Research Station at Crowley, Louisiana. On the following day, *B. glumae* suspension (OD600=0.1) was sprayed on the pretreated rice plants. Only the antagonistic bacteria showing the decreased symptoms in the greenhouse and having very strong antagonism in the *in vitro* test were selected for the field test rather than using all the antagonistic bacteria. One of the bacteria (RRB1045), which significantly minimized the disease symptoms in the greenhouse and had a strong antagonism in *in vitro* assays, was tested in the field by making different combinations with another strain of *Bacillus* spp. (RAB9), an antagonistic bacteria agaisnt *B. glumae* and *Rhizoctonia solani* in the laboratory condition. The RRB1045 was also combined with ascorbic acid (100 µM) in one treatment.

Disease scoring in the field was made 10 days after treatment of the antagonistic bacteria. Same scales as described in greenhouse test were used to determine the disease severity on rice panicles. Disease ratings were statistically analyzed by Tukey's honest significance difference (HSD) test (Tukey, 1975).

3.3 Results

3.3.1 Isolation of bacteria from rice rhizosphere and *in vitro* test against *B. glumae*

About 10,000 bacterial colonies were obtained from the rice rhizospheres, which were collected in June-August, 2013 from the Rice Research Station. Among them, 10 bacterial isolates showed inhibition regions when grown with *B. glumae*. Bacteria isolated from the rice rhizosphere were named as rice rhizospheric bacteria (RRB). In the medium plates, inhibition regions were clearly observed around the spot of the RRB, which blocked the growth of *B. glumae* (Figure 3.1). Area of growth inhibition zones of *B. glumae* on the medium plates were considered as the degree of antagonism of the corresponding antagonistic bacteria. All the bacteria tested showed significant antagonistic activities (Figure 3.2).

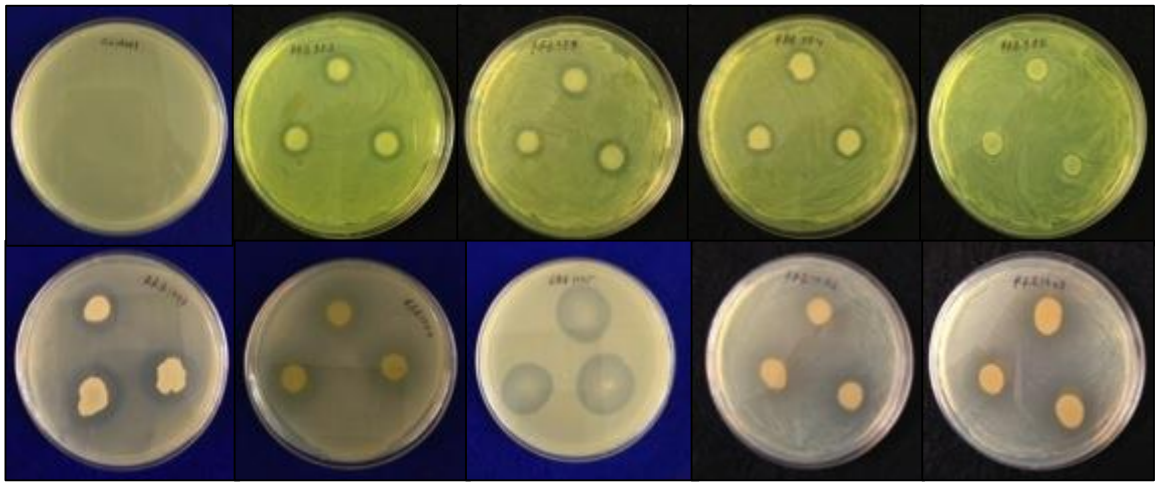

Figure 3.1. RRBs exhibit antagonistic characteristics against *B. glumae* by showing inhibition zones in laboratory condition.

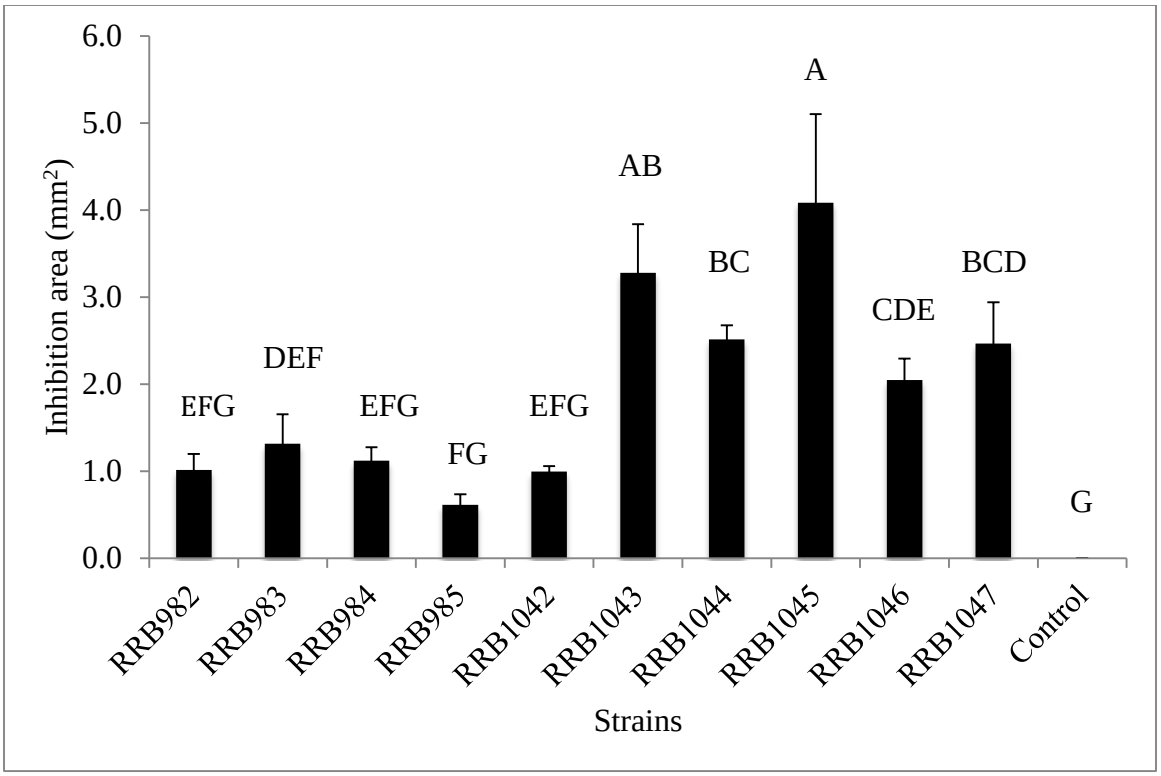

Figure 3.2. Growth inhibition area (mm^2) of *B. glumae* made by rice rhizospheric bacteria (RRB) on the medium plates in the laboratory condition. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with the same letter are not significantly different.

3.3.2 Identification of the antagonistic bacteria

Among 10 RRBs, seven were gram-positive and three were gram-negative bacteria, which were differentiated on the basis of KOH test. The 16S rDNA sequencing results reveled that six were members of *Bacillus*, one was *Paenibacillus* and three were *Pseudomonas* (Table 3.1). Identities of the bacteria listed in Table 3.1 were based on the 16S rDNA sequences, which were analyzed through Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI). In this study, RRB982, RRB983, RRB984, RRB985 and RRB1042 showed moderate antagonism against *B. glumae* in laboratory condition. However, in the same condition RRB1043, RRB1044, RRB1045, RRB1046 and RRB1047 showed strong antagonism against *B.*

glumae. In the phylogenetic analysis, bacteria were grouped on the basis of their identity and nearest ancestor (Figure 3.3). In the phylogenetic tree, bacilli (RRB982, RRB983, RRB984, RRB985 and RRB1043) were clustered in one group and the pseudomonads (RRB1044, RRB1046 and RRB1047) were clustered in one group. One *Paenibacillus* (RRB1045) was separated in a different group.

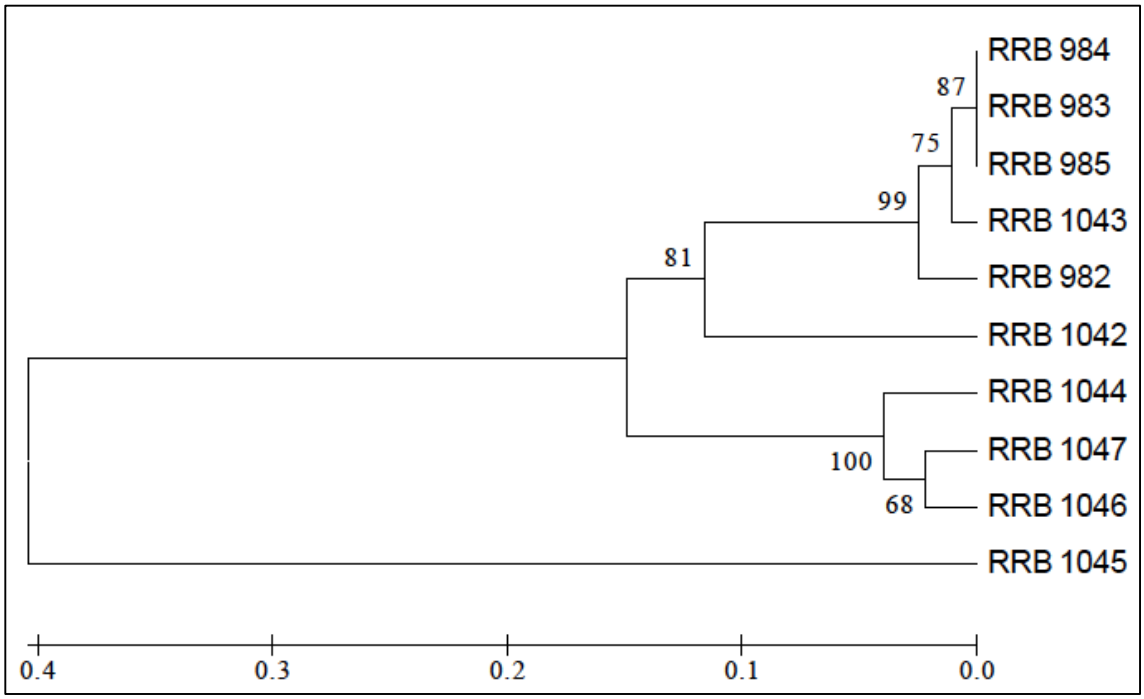

Figure 3.3. Phylogenetic tree resulted from analysis with the partial sequence information of 16S rDNA, which grouped the antagonistic RRBs into three major clusters. Phylogenetic tree was made by using neighbor-joining method and genetic distances were calculated using Kimura 2-parameter method.

Table 3.1. Rice Rhizospheric Bacteria (RRB), their identity, properties and degree of antagonism against *B. glumae* in laboratory condition. Species name in the bracket indicates the nearest species with higher identity percentage

Bacteria	Properties	Antagonism	Identity
RRB982	Gram positive	Moderate	*Bacillus subtilis*.
RRB983	Gram positive	Moderate	*Bacillus* sp. (*amyloliquefaciens*)
RRB984	Gram positive	Moderate	*Bacillus amyloliquefaciens*
RRB985	Gram positive	Moderate	*Bacillus subtilis*
RRB1042	Gram positive	Moderate	*Bacillus* sp. (*amyloliquefaciens)*
RRB1043	Gram positive	Strong	*Bacillus subtilis*
RRB1044	Gram negative	Strong	*Pseudomonas* spp. (*plecoglossicida*)
RRB1045	Gram positive	Strong	*Paenibacillus* sp. (*alvei)*
RRB1046	Gram negative	Strong	*Pseudomonas putida*
RRB1047	Gram negative	Strong	*Pseudomonas* sp. (*Putida*)

3.3.3 Greenhouse tests

In the greenhouse condition, all of the isolated RRBs suppressed the bacterial panicle blight in rice significantly. Some of the bacterial strains such as RRB983, RRB985 and RRB1047 reduced the disease rate up to 50 % as compare to the non-inoculated (Figure 3.4.). However, the disease suppression was not significant between RRBs.

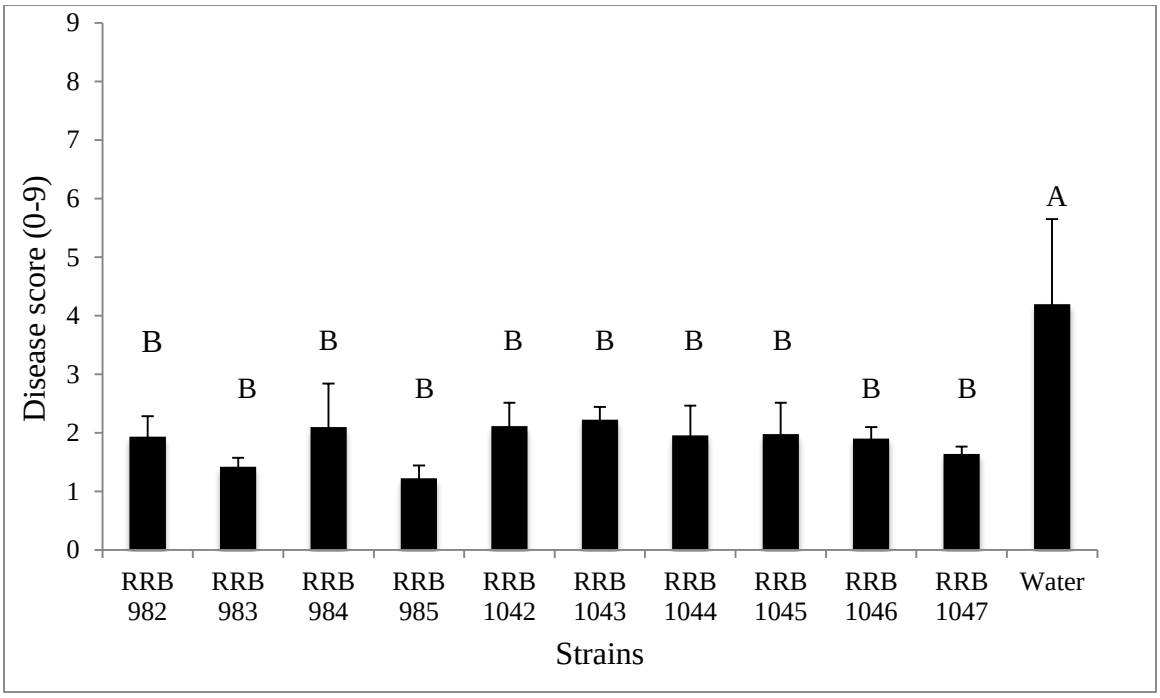

Figure 3.4. Rice rhizospheric bacteria suppressed bacterial panicle blight disease of rice in the greenhouse condition. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with the same letter are not significantly different.

3.3.4 Field test

RRBs showing disease suppression in the greenhouse also suppressed the panicle blight disease in the field. RRB1045, which was combined with other known suppressive bacteria and elicitors for the field treatment had a promising effect on the reduction of bacterial panicle blight in rice. RRB1045 in combination with ascorbic acid (AA) with the rate of 100 µM and Rice Associated Bacterium (RAB9, a bacterium isolated from rice plant, which was previously identified as an antagonistic bacterium against *B. glumae* in our laboratory) reduced the disease symptoms in rice panicles in comparison with the disease symptoms observed in the plants treated with only RRB1045 (Figure 3.5). Treatment of RRB1045 combined with AA (100 µM) suppressed the disease development better than the treatment of RRB1045 alone. But treatment of AA only

showed a similar level of disease suppression to the combined treatment of RRB1045 and AA, suggesting that RRB1045 does not add much to the disease suppression activity of AA (Figure 3.5). The combined treatment of RAB9 and RAB 6 (both bacteria were isolated from rice plants and were previously identified as antagonistic bacteria against *B. glumae* in our laboratory) did not show significant difference from individual treatments of RAB6 and RAB9 alone (Figure 3.5). As a whole in the field condition, RRB1044 showed highest disease suppression activity for rice bacterial panicle blight.

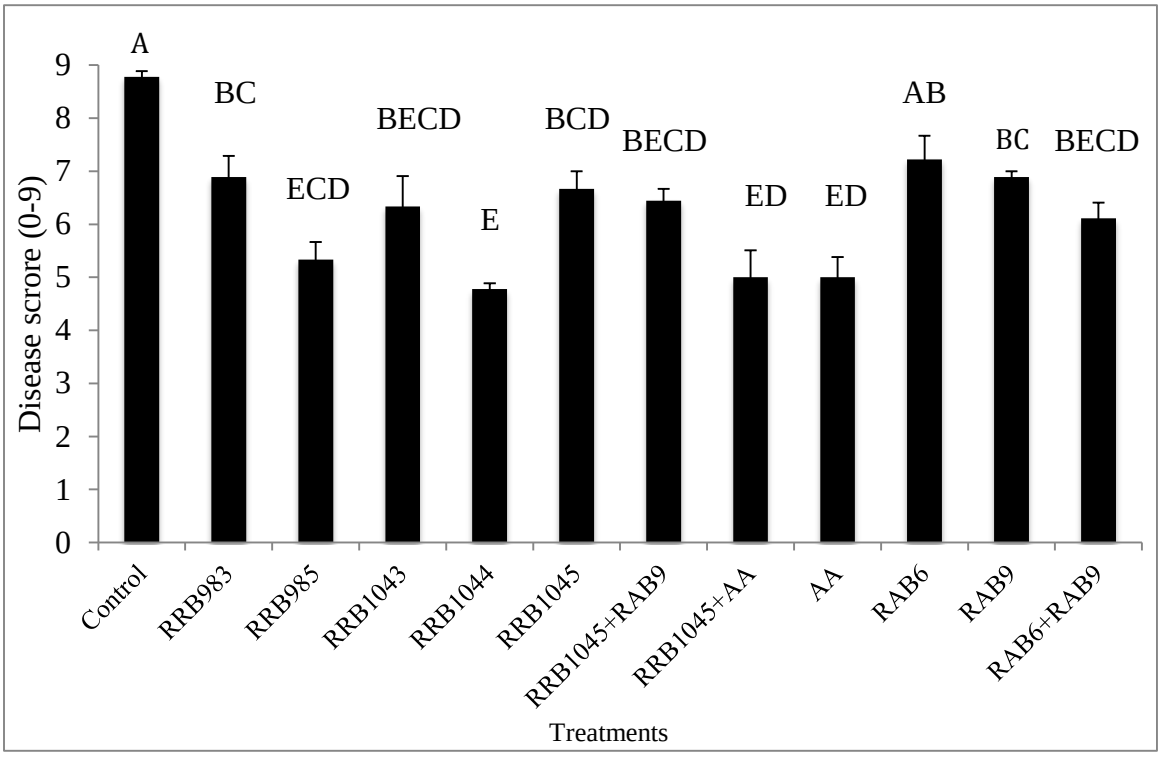

Figure 3.5. Suppression of bacterial panicle blight by rice rhizospheric bacteria in the field condition. Here, Ascorbic Acid (AA) was treated with 100 µM concentration and in co-treatment it was mixed with the respective bacterium culture and sprayed to the panicles of rice. Data were analyzed by using of Tukey's honest significant difference (HSD) test at P value 0.05. Means with the same letter are not significantly different.

3.4 Discussion

The interest in biological control of major crop diseases is increasing in agricultural enterprises as an alternative to chemical controls (Whipps, 2001). Research on biological control of rice pathogens started in the1980s with the identification, evaluation, and formulation of potentially important biological agents (Gnanamanickam, 2002). Among different biological agents, bacteria are considered as the best option because of their rapid growth, easy handling and good host-colonizing ability.

In most of the biological control strategies for crop diseases, bacterial antagonists *Bacillus* and *Pseudomonas* were considered as effective biocontrol agents for rice disease management (Desai *et al*., 2002). Bacilli are heat and desiccation tolerant, which is an important feature for useful biological agents to survive well in field conditions (Hokkanen *et al*., 2003). Similarly, pseudomonads are gram-negative bacteria having the ability to survive in low nutritional conditions and have excellent colonizing ability (Hokkanen *et al*., 2003). In our laboratory, Rice-associated bacteria showing antagonism against bacterial panicle blight and sheath blight have previously isolated and identified. Those bacteria belonged to the *Bacillus* group (Shrestha and Ham, unpublished). In this study, pseudomonads were isolated in addition to strains of *Bacillus* spp. as antagonistic bacteria from the rice rhizosphere. Those pseudomonads showed very distinct *B. glumae* growth inhibition zones in *in vitro* assay and significant suppression of panicle blight disease in greenhouse and field experiments.

In most cases, naturally occurring biological control in a field crop is likely to be the result of the interaction of multiple antagonists rather than from higher populations of a single antagonist (Raupach and Kloepper, 1998). In most of the investigations

conducted so far, mixtures of multiple biocontrol agents mimics natural system and enhanced the efficacy and reliability of disease control (Duffy and Weller, 1995) by allowing the combination of various mechanisms (Janisiewicz, 1988). In the field experiment conducted in this study, one of the treatments was the combination of RRB1045 and RAB9 to determine the effect of interaction on the disease suppression. In addition to that RAB9 and RAB6 were also combined and treated to the rice plants. Interestingly, it was observed that bacterial panicle blight was not significantly reduced in the rice plants treated with the combination of the antagonists in comparison to the treatments done singly (Figure 3.5).

Ascorbic acid (AA) is an important antioxidant and cellular reductant molecule, which is required for essential metabolic functions in animals and plants (Khan *et al*., 2011). In the plant, AA acts as immune-modulator and when it is applied in an appropriate concentration at the proper stage of plant development; many diseases are suppressed (Khan *et al*., 2011). It was hypothesized that the disease suppression would be higher in the plants treated with the combination of antagonists and AA than in the plants treated with an antagonist or AA alone. The result in this study suggests that the efficacy of disease suppression caused by antagonistic bacteria was enhanced by adding AA (Figure 3.5). However, the disease suppression was similar to the treatment of AA only. So, the effect of AA and antagonistic bacteria in the disease suppression was found to be independent.

It is very important to understand the mechanism of disease suppression for the successful application of biocontrol agents in disease management strategies. In this study bacteria belonging to genus *Bacillus*, *Pseudomonas* and *Paenibacillus* were

identified as potential source of biocontrol agents for management of bacterial panicle blight of rice. In addition, AA suppressed bacterial panicle blight as previously observed (Shrestha and Ham, unpublished). In future the selected antagonistic bacterial strains showing the strong antimicrobial activities can be used in genetic studies to identify the chemical compounds and bacterial gene responsible for their regulation. It is also the possible that the compound identified from those bacterial strains may have high commercial value for the crop disease management.

CHAPTER 4. CONCLUSIONS

Many research projects are being conducted to find out different management aspects to control bacterial panicle blight disease in rice. Chemical companies are seeking effective chemical formulations to control this disease in the field. However, no effective management practice has been identified for this disease. Molecular studies of the causal organism are very important to understand mechanisms related to the pathogenicity and virulence activities and to develop effective disease management tools. Similarly, physiological and genetic study of a causal organism will be helpful to make a foundation for the disease management.

Quorum sensing (QS) system, which is mediated by *lux* type *tofI* and *tofR* genes in *B. glumae*, is known to regulate its major virulence factors. However, most of the important virulence mechanisms, host resistance mechanism, and epidemiology of this pathogen are not well understood. Study on the negative regulatory systems of plant pathogenic bacteria will be a new kind of study even though it is not new for the animal pathogenic bacteria. In this study, *tepR* was identified as a negative regulator of major virulence factors in *B. glumae,* and it was characterized and confirmed through genetic approach and phenotypic observation. This study will be foundation for functional study of negative regulatory genes of *B. glumae* and other species of *Burkholderia*. For the management of disease in rice caused by this bacterium, different approaches such as development of the disease resistant varieties, chemical trials are being on study but effective management option is not identified yet. So, identification of rice rhizospheric bacteria having potential biocontrol ability to manage the bacterial panicle blight may be the good contribution from this thesis research for disease management.

In this study, we identified that the LuxO homolog, TepR, negatively regulates the production of the major phytotoxin of *B. glumae*, toxoflavin. The Δ*tepR* derivative of *B. glumae* 336gr-1 produced more toxoflavin than its parental strain and showed higher lipase and protease activities, indicating negative regulatory function of *tepR* on these phenotypes. However, the mutant did not produce any flagellar motility on the soft agar medium plate nor showed any flagellar structure under Transmission Electron Microscopy (TEM). Thus, *tepR* positively regulates the genes responsible for the flagella formation and flagellum-mediated motility in *B. glumae*.

Although the mechanism of TepR function still remains to be studied more, it may be possible that this gene regulates the virulence factors of *B. glumae* in the same way as LuxO proteins doe for bioluminescence and virulence in *Vibrio* spp. Since phosphorylated LuxO activates small RNAs (sRNAs) in *Vibrio* spp. and these sRNAs are responsible for repressing some important virulence gene expression, similar type of mechanism may exist in *B. glumae*. In-depth study of the TepR activities from phosphorylation to the production of sRNAs will be very important to understand the mechanism of its regulatory functions on different virulence factors. Since the *tepR* deletion mutant in our study did not show any AHL production, the t*epR* gene may also be related to the signal production and transduction system for the quorum sensing of *B. glumae*. AHL is known to be the major signaling factor of *B. glumae* for the production and transportation of toxoflavin. The result from this study suggests the presence of an unknown regulatory mechanism for toxoflavin production because the *tepR* deletion mutant produced more toxoflavin even if it does not produce AHL molecules. So, this result warrants a future study about the functional relationship of *tepR* with the quorum-

sensing gene, *tofI*, responsible for AHL molecule synthesis. It would be also useful to see the relationship between *tepR* and other known regulatory genes for bacterial virulence. The phenotypic characterization of a *tepR* deletion mutant performed in this study will be the foundation for the future studies of the regulatory network for the virulence of *B. glumae*.

Study on biological control agents for the management of bacterial panicle blight of rice is very important part of this thesis research. Adverse effects of agrochemicals can be minimized through the promotion of biological control methods for the crop disease management. In this study, different types of bacteria were isolated from the rice rhizospheric region and selected based on antagonistic characteristics against *B. glumae*. It is obvious that those bacteria are potential biocontrol agents to manage bacterial panicle blight and there is possibility that those bacteria may also have antagonistic activities against other plant pathogenic microbes (i.e. fungi and bacteria). So those bacteria can be a source of potential biocontrol agents for the management of other crop diseases. Future studies with the antagonistic bacteria may include identification of the genetic or chemical elements responsible for the antagonism to pathogenic microbes. Besides the disease suppression, those bacteria may be used to enhance the crop production because they might have some plant growth promoting ability.

In this way, better understanding of the virulence related genetic study of the rice pathogenic bacterium *B. glumae* and study of the potential biocontrol agents for controlling bacterial panicle blight disease of rice can help to develop effective disease management strategies for bacterial panicle blight.

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Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L., & Mekalanos, J. J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, *99*(5), 3129-3134.

APPENDIX A. RNA EXTRACTION FOR REVERSE TRANSCRIPTION (RT) PCR

The bacterial cultures were prepared by growing at 37^0C overnight. 1 ml of each bacterial culture was washed down by LB broth and resuspended in the equal volume of LB broth. About 10 µl of the suspension was inoculated in 10 ml of LB broth and incubated at 37°C. One ml of bacterial culture have optical density 1 at OD_{600} was centrifuged to make pellet and then it was frozen in liquid nitrogen for 5-10 s. The bacterial pellet was resuspended in an equal volume of TRIzol® Reagent (Invitrogen) and then, incubated in room temperature for 5 minutes. About 200 µl of chloroform was added to the mixture and allowed to incubate in room temperature for 3 minutes after thoroughly vortexing for 15 seconds. Then the phases were separated by centrifugation at a full speed for 15 minutes at 4°C. Then the upper aqueous layer was transferred to a fresh tube and incubated at the room temperature for 5 minutes after adding 500 µl of isopropanol. Then, the mixture was centrifuged at 4°C in full speed for 10 minutes. The supernatant was carefully removed and the RNA pellet was washed with 75% ethanol. The RNA pellet was dried and then the pellet was resuspended in 22 µl of RNase free water.

To remove residual DNA, DNA-free™ DNase Treatment and Removal Reagents (Ambion®) was used. To the RNA samples, 5 µl 10X DNase I Buffer and 1 µl DNase I was added and then the mixture was centrifuged shortly before incubating at 37^0C for about 20 min. Then 5µl inactivation reagent was added to the reaction mixture and then incubated at room temperature for 2 min. After that, the total RNA samples were spun down through centrifugation. The supernatant from the centrifugation contained total RNA, which was stored at -80°C. Removal of DNA was verified by PCR by using for the

rDNA spacer regions with specific primers of *B. glumae* (Takeuchi et al., 1997). For RT-PCR, cDNA, was generated by using a ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA).

APPENDIX B. 16S DNA SEQUENCES OF ANTAGONISTIC BACTERIA

>Consensus: RRB_982-fD1.ab1, RRB_982-rD1.ab1
CCAGATTCCTTACGGGAAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCCGCGTGATGATGAAGGTTTTTCGGATCGCCAAGC
TCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGCTTGGCTCTTGATCCG
GTACCTAACCAAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGTAATTATTGGGCGTAAAGGGCTCGCAGGGCG
GTATTCTTAAGTATGATGTGAAAGCCCCCCGGTTCAACCCGGGGATGGGTCA
TGTAAATTGGGGGGGATCTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGT
AGCGGTGAAATGCGTAGAGATGTGGAGGAACACCCGTGGCGAAGTTGCGAC
TCTCTGGTCTGTAACTGACGCTGAGGAGCTGAAAGCGTGGTGAGCGATACAG
GATTAGATCCCTGGTAGTCCACGCCGTACACGACTGAGTGCTAAGTGTTAGG
CGGTTTCCGCACCTTAGTGCTGCATGTCTAACGCATTGAAGCACTCCGCCTGG
GGAGTACGGTCGCAAGATTGAAACTCAAAGGAATTGGCGGGGGCCCGCACA
AGCGGTGGAGCATGAGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CGTGACATCCTCGACAATCCTAGAGATACGGACGTCCCCTTCCGGGGGCAGA
GTGACAGTTGGTGCATGGTTGTCGTCAGCTCGTGTAGTGAGATGTTGGGTAA
GTCCCGCAACGAGCGCAACCCTTGATAGTGCCAGCATTCGGCACAAGGTGAC
TGCCGGTCAAACCGGAGGAAGGTGGGGATGACGTCATCATCATGCCCCTTAT
GACCTGG

>Consensus: RRB_983-fD1.ab1, RRB_983-rD1.ab1
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GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGG
TGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGGTAGCCGACCTGAGAGGGTGATCGGCC
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AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
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CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGC
GTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAACACTCCG
CCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTCTGACTAATCCTAGAGATAGGACGTCCCCTTCGGGGG
CAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACA

GAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCT
CAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTA
ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTA
GGAGCCAGCCGCCGAAGG

>Consensus: RRB_984-fD1.ab1, RRB_984-rD1.ab1
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GCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTACCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTNGGATGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATA
GGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
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AAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC
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GGGGAGTACGGTCGCAAGACTGAAACTCAAGGAATTGACGGGGGCCCGCAC
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GTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCA
CTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
ATCATCATGCCCCTTATGCTGGGCTACACACGTGCTACAATGGACAGAACAA
AGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCC
AGCCGCCGAAGGGGGACAGATGATTGGG

>Consensus: RRB_985-fD1.ab1, RRB_985-rD1.ab1
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CAAGCGTTGCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGA
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GCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGT
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TGCGTGAAGCT

>Consensus: RRB_1042-fD1.ab1, RRB_1042-rD1.ab1
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TTTTTAGGATCGTAAAAGTCTCTGGAGTATCGCCAGGGAAGTCGTCTAGGAT
GGAGTACTGCTCTTAAGGTTGACGTCACCTGGGAAGAAAGCCCCGGCTAACT
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>Consensus: RRB_1043-fD1.ab1, RRB_1043-rD1.ab1
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GGGCTAATACCGGATCTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCT
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>Consensus: RRB_1044-fD1.ab1, RRB_1044-rD1.ab1
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>Consensus: RRB_1045-fD1.ab1, RRB_1045-rD1.ab1
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CGGGTCCCCGTCACTCCATTGAGTCGGTCTTGCAACCGTACTCCCCAGGCGGA
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CATTCGTTTACGGCGTGGACTACCAGGGTATTTCCTGTTTGCTCCCCACCCTTT
CGCGCCTCACCGTCAGTACAACCCAGAAGTCACCTTCCCCGGTGTTCCTCTCT
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ACAT

>Consensus: RRB_1046-fD1.ab1, RRB_1046-rD1.ab1

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GTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG
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CACGGTGTGATTCATGAC

>Consensus: RRB_1047-fD1.ab1, RRB_1047-rD1.ab1
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GCGGTCGGAATCGCTAGTAATTTTCAAATCAGAATGTCTGCTTGGTGAACTAC
GTCTCTCC

APPENDIX C. SOME OF THE BACTERIA SHOWING ANTAGONISTIC ACTIVITY AGAINST B. GLUMAE SHOWED ANTAGONISM AGAINST RICE SHEATH BLIGHT CAUSING FUNGI RHIZOCTONIA SOLANI IN LABORATORY CONDITIONS.

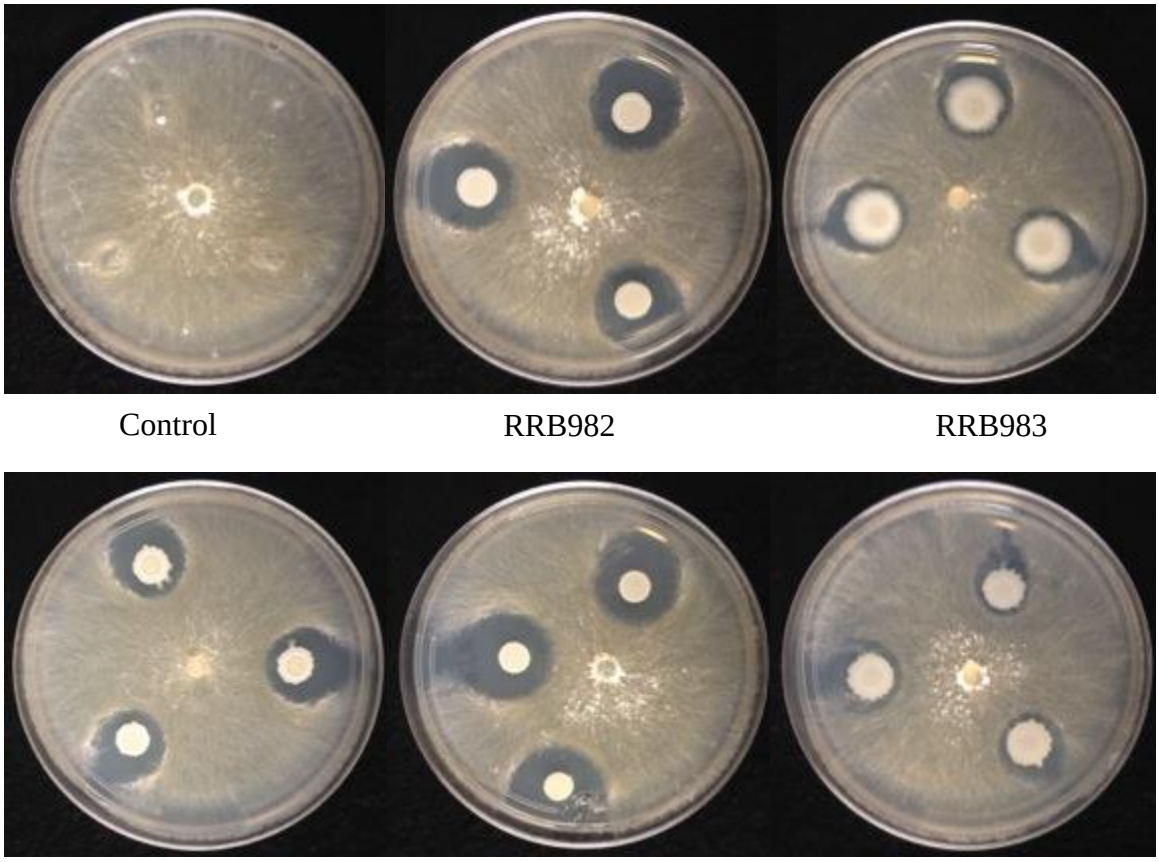

Figure. Rice rhizospheric bacteria showing antagonistic characteristics against *Rhizoctonia solani* in laboratory condition. RRB982, RRB983, RRB984, RRB985 and RRB1044 exhibited the inhibition zone in PDA plates when grew with *R. solani.*

APPENDIX D. RELATIVE VALUE OF TOXOFLAVIN PRODUCTION

Strains	OD_{260}				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	0.212	0.213	0.312	0.246	0.0574
LSUPB401	0.538	0.602	0.550	0.563	0.0340
LSUPB401 (pBB5-*tepR*)	0.237	0.240	0.301	0.259	0.0361
LB broth	0.001	0.000	0.000	0.000	0.0006

Strains	OD_{260}				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	0.630	0.640	0.570	0.613	0.0379
LSUPB401	0.847	0.923	0.863	0.878	0.0401
LSUPB401 (pBB5-*tepR*)	0.691	0.708	0.667	0.689	0.0206
LB broth	0.000	0.000	0.000	0.000	0.0000

Strains	OD_{260}				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	0.431	0.45	0.393	0.425	0.0290
LSUPB401	0.864	0.852	0.762	0.826	0.0557
LSUPB401 (pBB5-*tepR*)	0.451	0.400	0.436	0.429	0.0262
LB broth	0.001	0.000	0.001	0.001	0.0006

APPENDIX E. RELATIVE VALUE OF LIPASE ACTIVITIES

Strains	OD_{410}				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	0.450	0.470	0.500	0.473	0.0252
LSUPB401	0.980	0.900	0.950	0.943	0.0404
LSUPB401 (pBB5-*tepR*)	0.510	0.470	0.440	0.473	0.0351
LB broth	0.000	0.000	0.001	0.000	0.0006

Strains	OD_{410} (Undiluted)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	0.655	0.650	0.651	0.652	0.0026
LSUPB401	1.900	1.800	1.910	1.870	0.0608
LSUPB401 (pBB5-*tepR*)	0.651	0.660	0.680	0.664	0.0148
LB broth	0.001	0.000	0.002	0.001	0.0010

APPENDIX F. MACERATION AREA ON ONION SCALES

Strains	Maceration area (mm^2)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	91.85	105.98	60.45	86.09	23.30
LSUPB401	197.82	129.53	87.92	138.42	55.49
LSUPB401 (pBB5-*tepR*)	84.78	117.75	75.36	92.63	22.26
Control	0.00	0.00	0.00	0.00	0.00

Strains	Maceration area (mm^2)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	54.95	42.39	54.95	50.76	7.25
LSUPB401	75.36	138.16	117.75	110.42	32.03
LSUPB401 (pBB5-*tepR*)	60.45	60.45	65.94	62.28	3.17
Control	0.00	0.00	0.00	0.00	0.00

APPENDIX G. VIRULENCE ASSAY IN RICE IN GREENHOUSE

Strains	Disease score (0-9)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	5.7	6.0	6.7	6.1	0.51
LSUPB401	9.0	8.7	9.0	8.9	0.19
LSUPB401 (pBB5-*tepR*)	7.0	6.7	7.3	7.0	0.33
Control	0.0	0.0	0.0	0.0	0.00

Strains	Disease score (0-9)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	6.3	6.0	7.3	6.6	0.69
LSUPB401	8.3	8.3	8.7	8.4	0.19
LSUPB401 (pBB5-*tepR*)	6.7	6.0	7.0	6.6	0.51
Control	0.0	0.0	0.0	0.0	0.00

Strains	Disease score (0-9)				Standard deviation
	Rep I	Rep II	Rep III	Average	
336gr-1	3.71	4.00	3.50	3.74	0.25
LSUPB401	6.33	5.33	4.67	5.44	0.84
LSUPB401 (pBB5-*tepR*)	4.50	3.83	3.75	4.03	0.41
Control	0.00	0.00	0.00	0.00	0.00

APPENDIX H. HOMOLOGY OF TEPR AND LUXO

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B.glumae            1 MPHALIVEDDPNSLSGLTALLAADGFTVDTATSLAEARAALARTIPDVVL     50
                        :|||..:..:...:.|...|..::...:..:|..:|...|||::|
Vibrio              1 -----MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLIL     45

B.glumae           51 VDLNLPDGSGFDLLQHLPQPQQPNGGLPVIVLTGNATVESAIEGLRYGIW    100
                      :||.|||.:|.|:| |..:...|:  :|:|.:|.:.::::|:|.:|:|..
Vibrio             46 LDLRLPDMTGMDVL-HAVKKSHPD--VPIIFMTAHGSIDTAVEAMRHGSQ     92

B.glumae          101 DYLLKPVNIPRLRSLLARIPRPYELIEEVQALRTTLRKLG--RFGPLIGR    148
                      |:|:||....|||..:      ...|.:...|:......|  .:...||.
Vibrio             93 DFLIKPCEADRLRVTV------NNAIRKATKLKNEADNPGNQNYQGFIGS    136

B.glumae          149 SNAVQHVYDMIERNARSEAAVLVWGEAGTGKETTARALHDLSRRRKGPFI    198
                      |..:|.||..|:..|.|:|::.:.||:|||||..|.|:|..|:|...|||
Vibrio            137 SQTMQQVYRTIDSAAASSKASIFITGESGTGKEVCAEAIHAASKRGDKPFI    186

B.glumae          199 KFDCRAALQLPRVMELGNIVIESMLFGRERGSYGGAERREPGLFEQASGG    248
                      ..:|.|   :|:.:      |||.|||..:|::.||.....|..|.|.||
Vibrio            187 AINCAA---IPKDL------IESELFGHVKGAFTGAANDRQGAAELADGG    227

B.glumae          249 TLLLKDITALPLPIQESLLRALDSQSFRRIEGTTEVATDFRLI-ATSRRP    297
                      ||.|.::..:.|.:|..|||.:.:.:|:::..:...:.|.|.: ||:|.|
Vibrio            228 TLFLDELCEMDLDLQTKLLRFIQTGTFQKVGSSKMKSVDVRFVCATNRDP    277

B.glumae          298 SREALDHGTLREDLWMRLDAASIALPPLRARDDDMLQIAQAFVDDLNRQA    347
                      .:| :..|..||||:.||....:.|||||.|..|:::||.:.   |...:
Vibrio            278 WKE-VQEGRFREDLYYRLYVIPLHLPPLRERGKDVIEIAYSL---LGYMS    323

B.glumae          348 HDAGLGAVAKRIAPDFIRECLAYDWPGNVRELRDRVR------LAYEASG    391
                      |:.|...|  |.|.|.|....:|:||||||:|::.:|      ...|.:.
Vibrio            324 HEEGKSFV--RFAQDVIERFNSYEWPGNVRQLQNVLRNIVVLNNGKEITL    371

B.glumae          392 DFIES------LRANDGVFVPGAALSGSSVQVRVGTPLSDVEDLLIRATL    435
                      |.:..      :|.:...|:...::.|.:.     ||...|.:.|...:
Vibrio            372 DMLPPPLNQPVVRQSVAKFIEPDIMTVSDIM-----PLWMTEKMAIEQAI    416

B.glumae          436 DAVGGTRHRAAALLGISPKTLYNKLQRMKMN------    466
                      .|..|...|||..|.:||.|:|.|||.....
Vibrio            417 QACEGNIPRAAGYLDVSPSTIYRKLQAWNSKDEKQNV    453
```

```
Aligned sequences: 2
1: B. glumae
2: V. harvevi
Matrix: EBLOSUM62
Gap_penalty: 10.0
Extend_penalty: 0.5

Length: 487
Identity:     151/487 (31.0%)
Similarity:   242/487 (49.7%)
Gaps:          55/487 (11.3%)
Score: 581.5
```

VITA

Surendra Osti was born in Gorkha district of Nepal in March, 1988. He completed his higher secondary level education from Orchid Science College, Chitwan, Nepal. He started his undergraduate study in Agricultural Science in Institute of Agriculture and Animal Science, Rampur Chitwan in 2006 and he pursued Bachelor of Science in Agriculture in 2010 with majoring Plant Pathology. After finishing his bachelor, he did job as a plant doctor in SECARD, Nepal and voluntarily joined in NARC, Nepal in Division of Plant Pathology, Khumaltar, Kathmandu for a year. He was offered as an Exchange Visiting Scholar from University of Florida to do work as an intern in North Florida Research and Education Center, Quincy, Florida and worked there for three months (February, 2012 to April, 2012). He joined Louisiana State University as a graduate student under Dr. Jong Hyun Ham in May, 2012 to pursue Master's degree in Plant Health from Department of Plant Pathology and Crop Physiology. His Master's research project focused on Characterization of a σ^{54}-dependent response regulator, *tepR*, in the rice-pathogenic bacterium *Burkholderia glumae* and development of biocontrol strategies for bacterial panicle blight of rice. He also received a travel award recently from Department of Plant Pathology and Crop Physiology Graduate Student Association recently to attend annual meeting of American Phytopathological Society, 2014.