2011

A molecular genetic study on the TofI/TofR quorum-sensing system of Burkholderia glumae: the major pathogen that causes bacterial panicle blight of rice

Ruoxi Chen
Louisiana State University and Agricultural and Mechanical College, rchen5@lsu.edu

A MOLECULAR GENETIC STUDY ON THE TOFI/TOFR QUORUM-SENSING SYSTEM OF *BURKHOLDERIA GLUMAE*: THE MAJOR PATHOGEN THAT CAUSES BACTERIAL PANICLE BLIGHT OF RICE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

By

Ruoxi Chen
B.S., Shandong Agricultural University, China, 2008
December, 2011

Dedication

I would like to dedicate this theses to my parents: Tao Chen and Yinxia Zhang who always support and encourage me to be a better person; my aunt Min Chen and uncle Jianhua Zhang who inspired me to be a plant pathologist and helped me come to Louisiana State University.

Acknowledgements

I want to thank all the professors, colleges, and friends who helped with my study and living life. First of all, I am very grateful to all the generous help and support from my major adviser Dr. Jong Hyun Ham. He gave me this precious opportunity to study in molecular plant pathology area, offered great advice, spent time and patience to my work. I also want to thank my committee members Dr. Milton C. Rush and Dr. Rodrigo A. Valverde, who helped me with course and research work as well as the correction of this thesis. I appreciate all the teamwork I have with my lab mates, who always share good research experience with me: Inderjeet Kaur Hari Karki, Rebecca Melanson, Bishnu Shrestha and Felix Frances. I also want to give my gratitude to all other faculty, staff and students in my department, from where I have been learning, influenced and growing as a beginner in plant pathology.

Table of Contents

Abstract

Burkholderia glumae is the major causal agent of an economically important rice disease, bacterial panicle blight (BPB). The known virulence factors of *B. glumae* share the TofI/TofR quorum sensing system as their regulator. *tofI* and *tofR* genes encode the N-acyl homoserine lactone (AHL) synthase for the *B. glumae* quorum sensing signals, *N*-octanoyl homoserine lactone (C8-HSL) and *N*-hexanoyl homoserine lactone (C6-HSL), and the receptor for C8-HSL, respectively. To better understand the relationship between quorum sensing and known virulence factors (toxoflavin, flagella and lipase), as well as, putative virulence factors (i.e. extracellular polysaccharide), mutagenetic and phenotypic analyses were applied to this study. A technical breakthrough is the generation of a novel deletion mutation system-pBBSacB vector, which can effectively delete target genes from the genome and gives more reliable results. Quorum sensing gene deletion mutants were successfully created by using pBBSacB with a sucrose-sensitive counter selective marker, SacB. The parental strain 336gr-1 and its mutants have undergone a series of phenotypic observations and quantification tests for virulence changes. Toxoflavin and swarming motility were confirmed as the major virulence factors in 336gr-1, whereas lipase and EPS were not determined as critical for causing symptoms. The results confirmed the importance of quorum sensing system in expressing virulence, but also indicated that other regulators may be implicated in pathogenicity. Additionally, *orf1*, which is located between *tofI* and *tofR*, was postulated as a functional regulatory component.

Chapter 1. Literature Review and Introduction

1.1 Bacterial Panicle Blight and the Pathogen

Rice is an economically important crop that has been traditionally grown in Louisiana. Bacterial panicle blight (BPB) of rice, caused by *Burkholderia glumae* and *Burkholderia gladioli* (Nandakumar et al. 2009), has become one of the most important diseases on rice through Louisiana and the southern regions of United States. The major pathogen of this disease is *B. glumae*, whereas *B. gladioli* is less virulent (Nandakumar et al. 2009). Typically symptoms of panicle blight in Louisiana are seedling blight, sterile floret, sheath rot and grain rot (Shahjahan et al. 2000b; Tsushima 1996; Yuan 2004). Bacterial panicle blight was first described in Japan, in 1956 (Goto and Ohata 1956); within 30 years, this disease has been reported from many areas including East Asia, Southeast Asia and South America (Chien and Chang 1987; Cottyn et al. 1996; Shahjahan et al. 2000b; Zeigler and Alvarez 1989). In 1996, *B. glumae* was identified as the primary causative agent causing panicle blighting symptoms in the United States (Shahjahan et al. 2000b). In Louisiana, major epidemics of BPB occurred in 1995, 1998, 2000, and 2010, during which nighttime temperatures were warm and humid (Lariceman 2010; Shahjahan et al. 2000b). The loss caused by bacterial panicle blight could be as high as 70 percent, including reduced yield and poor milling (Groth and Hollier 2011). To make things worse, global warming has been making the environment more favorable for the infection of *Burkholderia glumae*. Despite the economic importance of *B. glumae*, the knowledge about this bacterium is still limited. But based on the facts we have already known, much more information about the virulence factors and the regulatory systems can be obtained by basic molecular biological and genetic studies. This information is critical for developing resistant varieties and controlling panicle blight on rice.

1.2 *Burkholderia* Species

The genus *Burkholderia* was initially identified as RNA homology group II of the genus *Pseudomonas*. In 1992, seven species in this group were proposed by Yabuuchi *et al* for transfer to the new genus, *Burkholderia* (Yabuuchi et al. 1992). *Burkholderia* species were first identified as soil dwelling bacteria, but the diversity of this genus has increased dramatically as more species have been described. Many *Burkholderia* species form pathogenic or symbiotic relationships with plants, whereas some species are closely related to human life (Coenye and Vandamme 2003). The type species, *B. cepacia* and other 16 related species are known as the *B. cepacia* complex (Bcc), which can also be isolated from humans (Vandamme and Dawyndt 2011). Bcc bacteria are notorious for being opportunistic pathogens, causing severe symptoms in people suffering from cystic fibrosis (Vandamme and Dawyndt 2011). The human pathogenic *Burkholderia* species also include the potential bioterrorist agents *B. mallei* and *B. pseudomalle*, causal agents of glanders and melioidosis, respectively (Galyov et al. 2010; Holden et al. 2004; Rajinikanth et al. 2008). On the other hand, agriculturally, Bcc species are considered highly beneficial because many produce antimicrobial compounds preventing plant diseases such as damping off, *Aphanomyces* root-rot or some postharvest diseases (Janisiewicz et al. 1991; King and Parke 1993; Mahenthiralingam et al. 2005; Parke and Gurian-Sherman 2001). Bcc species have bio-control potential as viable substitutes for chemical pesticides. In addition to the antagonistic effects they have on certain plant pathogens, Bcc species can also promote plant growth and plant production by fixing nitrogen (Luvizotto et al. 2010). *B. glumae* is closely related to Bcc bacteria and the detailed study of *B. glumae* will provide more information regarding the virulence of other important species.

1.3 *Burkholderia glumae* and its Virulence Factors

B. glumae are aerobic, non-fluorescent, and rod shaped Gram-negative bacteria. They achieve motility by means of a polar flagellar tuft. (Saddler 1994). Their optimum growth temperature is 38~40 ℃, and *B. glumae* can even live at 50 ℃ (Nandakumar et al. 2009). *B. glumae* is known as a plant pathogen. In 2007, the *B. glumae* strain AU6208, was isolated from a surgical specimen culture of an 8-month-old boy with chronic granulomatous disease (CGD) (Weinberg et al. 2007), which was the first and the only report regarding the pathogenicity of this bacterium on a human.

A typical characteristic of *B. glumae* is the production of bright yellow pigments, toxoflavin and fervenulin, which were identified as the inducers for rot symptoms. (Iiyama et al. 1995; Kim et al. 2004; Nagamatsu 2002). The more important substance is toxoflavin, which is also responsible for chlorosis on the infected grains (Suzuki et al. 2004). *B. glumae* does not produce much toxoflavin at temperatures below 28 ℃ and the optimal temperature for toxoflavin production is 37 ℃ (Matsuda and Sato 1988); thus, this may explain why bacterial panicle blight devastatingly affects geographic areas such as Louisiana which experiences high average temperatures during the rice growing season. Besides that, *B. glumae* also causes bacterial wilt on tomato, sesame, perilla, eggplant, hot pepper and 20 other plant species (Jeong et al. 2003).

Toxoflavin production is conferred by a gene cluster operon *toxABCDE* (Suzuki et al. 2004). The mechanism of toxicity might be toxoflavin activating the producing of hydrogen peroxide (Latuasan and Berends 1961). Like toxoflavin, there are other virulence factors critically involved in the pathogenicity of *B. glumae* as well. In 2007, it was found that a *lipA* (encoding LipA lipase) mutant of the clinic strain AU6208 was no longer pathogenic to rice, indicating that the lipase is another important virulence factor (Devescovi et al. 2007). The

possible roles that lipase plays in causing blighting are degrading the xylan or epicuticular waxes to destroy the barrier system for the plant cells (Devescovi et al. 2007). In the same year, the role of flagella in virulence was demonstrated for *B. glumae* (Kim et al. 2007). QsmR, as an IclR-type transcriptional regulator, is important for flagellum formation and regulated by quorum sensing and the non-motile bacteria could produce toxoflavin, but lost the capacity to cause symptoms (Kim et al., 2007). It was confirmed in 2009 that QsmR upregulated another virulence factor KatG, a major catalase that protects bacterial cells from visible light (Chun et al. 2009). As formerly described, toxoflavin could produce hydrogen peroxide, which was not only toxic to the plant cells, but also to the bacterial cells themselves (Latuasan and Berends 1961). Thus, the fact that catalase could help the pathogen survive in light and in an aerobic environment makes it necessary for full virulence (Chun et al. 2009).

Type III secretion system (T3SS) is recognized as the infection apparatus through which the proteinaceous virulence factors (type III effectors) are directly secreted from the bacterial cell into the cells of the host organism (Galan and Wolf-Watz 2006). T3SSs are encoded by many bacterial species that are pathogenic on plants and animals, including humans (e.g. *Burkholderia pseudomallei* and *Burkholderia mallei*) (Cornelis and Van Gijsegem 2000; Galyov et al. 2010). T3SS can also elicit the hypersensitive response (HR) on resistant host or non-host plants (Oh et al. 2007). It was proved that *B. glumae* could elicit HR on tobacco and the T3SS defective mutant lost full virulence (Kang et al. 2008), which made T3SS become another virulence factor of *B. glumae*. No effector has been identified secreted by T3SS in *B. glumae* yet (Ham et al. 2011). T3SS has already attracted a lot of attention, and progress about understanding T3SS functions in different bacteria are being made every day, and more research about T3SS in *B. glumae* pathogenicity should be published soon.

Extracellular polysaccharide (EPS) is considered to be another potential virulence factor in *B. gluame* in this study. On casamino acid peptone glucose (CPG) medium (Schaad et al. 2001), virulent *B. glumae* strains can produce EPS which give the bacterial colony a mucoid surface. EPS is known as a virulence factor for some wilting bacteria (e.g. *Erwinia amylovor*a, *Pantoea stewartii* and *Ralstonia solanacearum*) that cause symptoms in xylem of the plant (Langlotz et al. 2011; Saile et al. 1997). In addition to that, *B. glumae* can also cause wilting symptoms in over 20 species of plants (Jeong et al. 2003). The production of EPS in Bcc complex can be induced on onion extract agar (Bartholdson et al. 2008). There is evidence that EPS is a putative virulence factor in *Burkholderia* species. For example, EPS production in mice lungs could help mucoid *Burkholderia cenocepacia* isolates survive leukocytosis and enhance the virulence (Conway et al. 2004). Cepacian, as the major EPS produced by most of the clinical isolates of the *Burkholderia cepacia* complex, contributes to pathogenicity in hosts and to tolerance to desiccation and metal ion stress (Ferreira et al. 2010).

1.4 Quorum Sensing

Quorum sensing is a cell-to-cell communication system that bacteria use to collectively behave in a way that resembles a multicellular organism (Eberl and Riedel 2011). Bacteria can use diffusible signal molecules known as autoinducers to release and sense intercellular information (Atkinson and Williams 2009). The quorum sensing dependent genes' transcription can be turned on or off to make different responses to enable adaptation to environment changes (Di Cagno et al. 2011). Autoinducers are constantly produced and secreted by bacteria, but they are also diluted in the extracellular environment and would not effectively control target genes until the bacterial population passes a certain threshold, or quorum (Ng and Bassler 2009). At the point of quorum, target genes (e.g. virulence genes) can elicit the defense reaction in the host, but

with the monitoring by quorum sensing regulation, the host would not respond until the bacterial concentration is already sufficient enough to overcome the defense response function (Mae et al. 2001). Quorum sensing pathogens can manipulate and inhibit the plant defense system, and that is why some people called quorum sensing a "stealth mode" (Liu et al. 2008).

Bacteria have cognate receptors that can specifically bind autoinducer molecules diffusing from the extracellular environment at the quorum point (Ng and Bassler 2009). The autoinducer/receptor complex activates the synthesis of a group of genes which encode various types of physiological processes: e.g. bioluminescence, biofilm formation, motility, pathogenicity and competence development (de Kievit and Iglewski 2000; Fuqua et al. 2001; Hamoen et al. 2003; Kohler et al. 2000; Ng and Bassler 2009; Singh et al. 2000; Solomon et al. 1996).

Quorum sensing is a hierarchichal mechanism with many intertwined regulators coordinating together to adopt their population behaviors. There are at least six types of identified quorum sensing signals, and the most common ones are: *N*-acyl homoserine lactones (AHLs), produced by Gram-negative bacteria e.g. *B. glumae*; autoinducing peptides (AIPs), produced by Gram-positive bacteria e.g. *Bacillus subtilis*; and autoinducer II (AI-2), the intra and inter-species cross talk signal e.g. between *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (Eberl and Riedel 2011; Fong et al. 2001). Besides, Bcc bacteria were found to produce other types of autoinducer-diffusible signal factors (DSFs) (Boon et al. 2008; Deng et al. 2010) also for intra- or inter- species communication. Additionally, one type of quorum sensing signal can be expressed in diverse kinds of bacteria, and one bacterial strain can harbor more than one type of signaling system.

1.5 AHL/LuxI/LuxR Regulator System

AHLs are low molecular weight lipids which contain homoserine lactone rings and acyl chains of varying lengths (Fuqua et al. 2001). The different length, degree of substitution and saturation contribute to the specificity of each AHL molecule, which is distinguished by quorum sensing bacteria, especially by strains that produce multiple AHLs (Fuqua et al. 2001). The synthase and receptor for AHL signaling are the LuxI and LuxR family proteins, respectively. LuxI type protein binds to *S*-adenosylmethionine (SAM) to initiate the syntheses of AHL molecules; another donor is fatty acid biosynthetic precursor acylated-acyl carrier protein (ACP) (Fuqua et al. 2001; More et al. 1996). After formation of the amide bonds, AHL, holo-ACP and 5'-methylthioadenosine are produced (Watson et al. 2002). When the intercellular density of accumulated AHLs reach a critical level, the cognate receptor LuxR will bind on the AHL and regulate target genes' expression, including *luxI* gene (Eberl and Riedel 2011).

The LuxI/LuxR regulator system is considered the fundamental model for Gram negative bacterial quorum sensing. This system was first discovered from the characterization of quorum sensing in *Vibrio fischeri*, a luminous symbiont in marine fish (Eberhard 1972; Fuqua et al. 1994). However, even though the critical paradigm is shared by all, different AHL-type quorum sensing systems have been reported. Some important plant pathogenic bacteria have been intensively used as models for studying the LuxI/LuxR quorum sensing system, such as: the EsaI/EsaR system in wilt-causing *Pantoea stewartii* regulates expression of major virulence factor EPS (von Bodman et al. 1998) ; the TraI/TraR system of the crown gall pathogen *Agrobacterium tumefaciens* activates translocation of the Ti plasmid to the plant cells (Hwang et al. 1994); the AhlI/AhlR system in *Pseudomonas syringae* contributes to symptoms caused by internal tissue maceration within the host (Quinones et al. 2005). With the emerging importance

of pathogenic *Burkholderia* species, TofI/TofR quorum sensing virulence regulation system in *B. glumae (*Kim et al. 2004) has become a popular and practical model for investigating pathogenicity in plants as well as in humans.

There are various LuxI/LuxR quorum sensing systems in *Burkholderia* species, and the predominant one probably is CepI/CepR global regulator (Gotschlich et al. 2001; Leo 2006). The CepI/CepR system was the first quorum sensing system identified in the *Burkholderia* genus, for siderophore ornibactin negative regulation and extracellular protease positive regulation in *B. cepacia* (Lewenza et al. 1999). It was also found that expression of plasmid-borne *cepR-lacZ* increased two-fold in *B. cepacia* CepR mutants, which indicated that the *cepR* gene had negative self-autoregulation property (Lewenza and Sokol 2001). A similar expression pattern was found in *B. cenocepacia* (Lewenza and Sokol 2001) .

It is not uncommon for *Burkholderia* species to have multiple LuxI/LuxR quorum sensing systems, e.g. *B. cenocepacia* has CepI/CepR and CciI/CciR in a genomic island both contributing to virulence (Baldwin et al. 2004; Sokol et al. 2003), and an orphan CepR2 LuxR homolog autoregulator which positively regulates siderophore pyochelin (Malott et al. 2009). *B. vietnamiensis* has CepI/CepR and BviI/BviR (Conway and Greenberg 2002; Lutter et al. 2001), but the target regulon has not been well studied yet. Some species such as *B. pseudomallei* and *B. mallei* have even more complicated quorum sensing systems. *B. mallei* has two Lux homolog pairs - BmaI1/BmaR1 and BmaI3/BmaR3, and two orphan putative LuxR regulators, BmaR4 and BmaR5, all of which six Lux type proteins are required for full virulence (Duerkop et al. 2008; Duerkop et al. 2007). *B. pseudomallei* has two orphan LuxR homologs and three BpsI/BpsR pairs, and two of the characterized BpsI/BpsR groups contribute to bacterial biofilm (Duerkop et al. 2007; Song et al. 2005).

1.6 Quorum Sensing System in *Burkholderia glumae*

The quorum sensing system of *B. glumae* was first identified in 2004, regulating the toxoflavin synthesis operon *toxABCDE* and transport operon *toxFGHI* (Kim et al. 2004; Shingu and Yoneyama 2004; Suzuki et al. 2004) . Kim et al. (Kim et al. 2004) used biosensor and chromatography to determine the existing autoinducers in *B. glumae* and found two LuxI type compounds C8-HSL and C6-HSL. After cosmid library screen using an *Agrobacterium* indicator, a corresponding clone was selected, which had *tofI* gene encoding a 22.4 kDa LuxI homolog protein and *tofR* gene encoding a 26.6 kDa LuxR homolog protein (Kim et al. 2004). The C8-HSL and TofR complex could activate the toxoflavin production regulator ToxJ which had the *lux* box-like construct in its upstream of encoding DNA; then indirectly, bacteria could monitor the toxflavin expression in the quorum sensing manner (Kim et al. 2004). This research group also confirmed that LysR-type activator ToxR is another toxoflavin regulator and requires toxoflavin as co-inducer (Kim et al. 2004). In 2009, Kim group used physical evidences to confirm their results obtained from genetic evidences, and provoked the simultaneous binding of ToxR and ToxJ to regulatory region the *tox* operons (Kim et al. 2009).

In this study, toxoflavin has been used as the main phenotypic marker for the mutational genetic study of TofI/TofR quorum sensing system in *B. glumae*. Deletion mutation of *tofI* and *tofR* genes has been made and the regulatory roles of quorum sensing genes on toxoflavin production in *B. glumae* strain 336gr-1 have been investigated. However, current data reveal some differences with the results from the Kim group: the TofI and TofR deficient mutants did not completely loose toxoflavin production, which suggested the possibility of other related regulators. Until now, only quorum sensing and ToxR were reported function in the regulatory

network for toxoflavin in *B. glumae*, but in this project, the existence of other regulators of toxoflavin was postulated.

As formerly mentioned, besides toxoflavin, other virulence factors such as lipase, flagella, and catalase are also proved to be regulated by quorum sensing. In 2007, Devescovi *et al.* (Devescovi et al. 2007) demonstrated that TofI/TofR quorum sensing deficient mutant of clinical isolate *B. glumae* AU6208 showed substantially reduced virulence on rice, which was most probably due to the down regulation of the virulence factor lipase (Devescovi et al. 2007). The same year, after the major virulence factor role of toxoflavin was proven, the Kim group again found that the aflagellate quorum sensing mutants highly lost their pathogenicity ability on rice (Kim et al. 2007). C8-HSL/TofR complex activated an IcIR type regulator QsmR by binding to the *qsmR* gene promoter region, and QsmR in turn regulated the expression of flagella genes *flhDC* (Kim et al. 2007). The last detail studied virulence factor, catalase, was also identified as indirectly regulated by quorum sensing through QsmR (Chun et al. 2009). Recently, Goo *et al.* applied proteomic analysis to search for proteins under the control of quorum sensing. There were as many as 79 proteins, including previously known quorum sensing-dependent proteins, differentially expressed between wild type BGR1 and the *tofI* mutant (Goo et al. 2010).

Phenotypic tests were also performed to all the mutants to determine the regulatory status of targeted genes when compared to the wild type strains. The role quorum sensing plays in the expression of the above described virulence factors (toxoflaivn, EPS, flagella and lipase) and the putative virulence factor EPS were characterized. The virulence assay was done by the onion maceration test to all the *B. glumae* derivative strains, because of its high efficiency. It was determined that 1,274 out of 1,290 Bcc onion rhizosphere isolates showed water soaking or maceration symptoms on onion (Jacobs et al. 2008). Considering the close phylogenetic

relationship *B. glumae* has with Bcc strains, our lab also tried using onion as an alternative host for pathogenicity tests, and it was proved fairly accurate (the correlation co-efficiency r^2 between the degree of maceration and disease severity on rice is 0.752) (Karki and Ham, unpublished). Initial data about the pathogenicity of the quorum sensing deficient mutants was obtained.

1.7 Goals and Objectives

The main goal of this study is to help develop a better understanding of the regulatory and virulence systems of *B. glumae*. It will contribute to the developing of new disease management methods for BPB. The strategies that I am using may add valuable information to the current knowledge of molecular genetics of *Burkholderia* spp. Furthermore, it will help build useful information models for the other human pathogenic and environmentally beneficial bacteria from the genus *Burkholderia*, and eventually help to develop effective treatments for human diseases and protect natural resources.

Chapter 2. Materials and Methods

2.1 Bacterial Strains and Plasmids List

Table 1 The bacterial strains and plasmids used in these experiments

Strain or Plasmid	Characteristic	Reference
Escherichia coli (*E. coli*)		
DH10B	Competent cells for broad host vector	(Grant et al. 1990)
DH5α	Competent cells for broad host vector	(Grant et al. 1990)
Sλ17-1pir	Competent cells for suicide vectors, Sm^r/Tp^r	(Simon et al. 1983)
Plasmid		
pRK2013::Tn*7*	Conjugation helper strain	(Figurski and Helinski 1979)
pKNOCK-Km	Broad host conjugative suicide vector, Km^r	(Alexeyev 1999)
pKNOCK-Gm	Broad host conjugative suicide vector, Gm^r	(Alexeyev 1999)
pSC*tofI*U	545 *bp* topo clone of upstream flanking region of *tofI*	This study
pSC*tofI*D	512 *bp* topo clone of downstream flanking region of *tofI*	This study
pSC*tofR*U	426 *bp* topo clone of upstream flanking region of *tofR*	This study
pSCtof*R*D	892 *bp* topo clone of downstream flanking region of *tofR*	This study
pKGm*tof*I	462 *bp tofI* internal fragment of 336gr-1 was inserted to pKNOCK-Gm at *Eco*RI	(Devescovi et al. 2007)
pKKm*tofI*	462 *bp tofI* internal fragment of 336gr-1 was inserted to pKNOCK-Km at *Eco*RI	(Devescovi et al. 2007)
pKKSacB	2.7 *kb sacB* gene was inserted to pKNOCK-Km vector at *Pst*I and *Bam*HI	This study

Table 1 continued

pKKSacBΔ*TofI/tofR/orf1*	pkkSacB containing *TofI/tofR* flanking region clone at *Spe*I	This study
pKKSacBΔ*TofI*	pkkSacB containing *TofI* flanking region clone at *Sal*I and *Kpn*I	This study
pKKSacBΔ*TofR*	pkkSacB containing *TofR* flanking region clone at *Spe*I	This study
Burkholderia glumae		
336gr-1	Wild type strain and the causative isolate of bacterial panicle blight of rice in Crowley, LA	Provided by Dr. Milton C. Rush and A.K.M. Shahjanhan
LSUPB214	336gr-1 *tofI*::Pknock-Km	This study
LSUPB220	336gr-1 *tofI*::Pknock-Gm	This study
LSUPB145	336gr-1 Δ*tofI*	This study
LSUPB139	336gr-1 Δ*tofI/tofR/orf1*	This study
LSUPB169	336gr-1 Δ*tofR*	This study
LSUPB201	336gr-1 Δ*tofI*/Δ*tofR*	This study
Chromabacterium violaceum		
Chromabacterium violaceum CV026	AHL production indicator strain	(McClean et al. 1997)

2.2 Recombinant Molecular Techniques

PCR reaction was performed in a 25 ul reaction mixture containg: H_2O 16.5µl, 10x Taq polymerase buffer 2.5µl (including $MgCl_2$), 10mM dNTP 0.5µl, Taq polymerase 2µl, template DNA 1µl, 10 µM forward primer 1µl, 10 µM reverse primer 1µl, dimethyl sulfoxide (DMSO) 0.5µl. The PCR conditions are listed in appendix. PCR products used for cloning were purified by QuickClean 5M PCR Purification Kit (from GenScript) and Topo-cloned using Strata CloneTM PCR cloning kit (from Stratagene). The pSC-A-amp/kan vectors containing the

expected size of homologous DNA inserts were sent out for sequencing in Genelab inVeterinarian School of LSU, using M13 forward and reverse primers. The genomic DNAs for the wild type or the mutant strains were extracted using GenElute^{TM} Bacterial Genomic DNA kit (from SIGMA). The plasmid DNA was extracted by an alkaline lysis method and followed by phenol extraction and ethanol precipitation. All the restriction digestion reactions were done as recommended by the supplier (BioLabs and invitrogen). The electrophoresis of DNA products was performed at ~120 voltages on 0.7% agarose gel. The electroporation for transforming *E. coli* competent cells was performed under 1.5 KV, between 2 μl ligated DNA and 25 μl competent cells. To extract the DNA from agarose gel, the GenElute^{TM} Gel extraction kit (from SIGMA) was used. The concentrations of the purified DNA were measured using NanoDrop DN-1000 Spectrophotometer. The genomic library of *B. glumae* 336gr-1 was created previously in our lab.

2.3 The Growth Condition of the Studied Strains

For normal growth, all the strains were inoculated on Luria-Bertani (LB) agar or broth (25 g LB broth, 18 g agar, and 1 L water) at 37 ℃. LB sucrose plates containing 30% sucrose were used to select the mutants with secondary recombination occurred in the homologous flanking region. For the toxoflavin and EPS expression test, King's B (KB) (20 g peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H2O$, 15 ml glycerol, 18 g agar, and 1 L water), CPG agar medium (1.0 g casamino acid, 10 g peptone, 10 g glucose, 18 g agar, and 1 L water), and Congo red medium (25 g LB, 18 g agar, 0.1 g Congo red, and 1 L water) were used for observation. To determine the growth and toxin production level of different strains, various treatments used for each *B. glumae* variant were: LB, LB broth without AHL signal; L6, LB with 1 μM C6-HSL; L8, LB with 1 μM C8-HSL; and L68, LB with 1 μM C6-HSL and 1 μM C8-HSL. Lipase media (10 ml

Tween 20, 25 g LB broth, 18 g agar and 1 L water) were used for lipase production observation. For toxoflavin, EPS and lipase producing tests, the cultured media were incubated at 37 ℃. The SWA (swarming) and SWI (swimming) plate containing 0.4% agar were used for the motility test of the bacterial strains according to the reported methods with some modifications: the LB overnight cultures were washed twice by fresh LB and inoculated 5 μl onto 0.4% agar. The cultures were incubated 48 hours at 37 ℃ for SWI and 30 ℃ for SWA (Kim et al. 2007). The antibiotics and the working concentration used in this study are: Ampicillin (Amp)-100 μg/ml, Kanamycin (Km)-50 μg/ml, Nitrofurantoin (Nit)-100 μg/ml, and Gentamycin (Gm)-20 μg/ml.

2.4 Disruptive Mutation of the *tofI* Gene

Internal homologous fragments in the center of *tofI* gene were amplified using primers tofI-vv1 and tofI-vv2 (Appendix) and cloned into pKNOCK-Km and –Gm conjugative suicide vectors. The resultant constructs pGmtofI and pKmtofI were confirmed by colony PCR using the same primers and restriction digestion analysis. The verified disruptive constructs were electroperated into competent cells of *E. coli* S17-1λ*pir*. Conjugation was performed as below: a) Grow overnight the donor (pKNOCK derivative), the recipient (*B. glumae* 336gr-1) and the helper strains (*E.coli* pRK2013::Tn*7*). b) Mix them in 1: 1: 1 ratio (500 μl donor + 500 μl recipient + 500 μl helper strain; 1 ml donor, 1 ml recipient and 1 ml helper were taken separately as controls). c) Centrifuge at 13, 2000 rpm for 1 min, discard the supernatant and resuspend the pellet in 50 μl LB broth. Spot culture on LB plate and incubate at 30 °C overnight. d) Scratch the culture and resuspend the bacteria into 1 ml LB broth, then plate on LB Nitro/Km or Nitro/Gm. Spread each plate with 100 μl of the suspension. Incubate the plates at 30 ℃ for two days. The parental wild type strain 336gr-1 has natural nitrofurantoin resistance, so nitrofurantoin and antibiotics expressed by individual pKNOCK vector were used to make the selective plates.

2.5 Construction of the pKKSacB Vector

The pKKSacB suicide vector was generated by research associate Inderjeet Kaur through subcloning of a *sacB* gene from pL3Sac to pKNOCK-Km suicide vector at *Pst*I and *Bam*HI restriction sites. pL3Sac, kindly donated by Dr. T. Danny, is a derivative of pLAFR3 and contains a copy of *sacB* gene around 2.7 *kb*. First, it was confirmed that the *B. glumae* cells in which *sacB* gene was expressed could not survive in sucrose containing medium. The newly constructed pKKSacB containing the 4.9 kb *sacB* gene was then used to make deletion mutation of the quorum sensing genes, using 30% sucrose as the selection marker.

2.6 Allelic Exchange Mutagenesis Using pKKSacB Vector

The PCR programs used for the target deletion mutation and confirmation are listed in Appendix. All of the allelic exchange mutagenesis work was performed with the collaboration with research associate Inderjeet Kaur. Δ*tofI* (LSUPB145), Δ*tofR* (LSUPB169), and Δ*tofI*/Δ*tofR* (LSUPB201) quorum sensing deficient mutants were obtained through double crossover recombination in their flanking region. The open reading frame (ORF) length of *tofI* and *tofR* are 612 *bp* and 720 *bp*, respectively, and separated by 796 *bp* DNA fragment (Kim et al. 2004). The ORF encoding one hypothetical protein within the 796 *bp* DNA fragment was tentatively named *orf1* and also deleted in wild type to get Δ*tofI*/*tofR*/*orf1* (LSUPB139) mutant. As a start for deletion mutation, the flanking regions (500~800 *bp*) of the target genes were amplified using primers with desired restriction site added as shown in Appendix. The flanking fragments for different target genes were all ligated at primer carrying the *Bam*HI site. The flanking region of *tofI* was subcoloned to entry vector pKKSacB at *Sal*I and *Kpn*I sites. The flanking region of *tofR* was subcoloned to pKKSacB at *Spe*I site.The upstream flanking region of *tofI* and downstream flanking region of *tofR* which were used to remove *tofI/tofR/orf1* were inserted at *Spe*I site in

pKKSacB vector. As described previously in 2.4, conjugation was induced between *B. glumae* 336gr-1 and the transformed *E. coli* cells. The selected conjugant colonies from LB/Km/Nitro medium were inoculated into LB broth, and grown overnight at 30 ℃. The overnight culture was then spread on LB sucrose plate and incubated at 30 ℃ for 48 hours. The growing colonies were transferred onto LB and LB/Km plates, and those that grew on LB plate but not on LB/Km plate were selected as mutants. Genomic DNAs of the mutants were extracted and confirmation PCR (Appendix) was performed using primers corresponding to the deleted region using the wild type genomic DNA as control.

2.7 AHL Signal Recovery Effect Test and AHL Production Assay

To furthermore illustrate the importance of the mutated genes, the AHL signal recovery effect on mutants was examined. The mutant and wild type strains were inoculated into LB broth in test tubes or onto LB agar plates. At the same time, artificial C6-HSL and C8-HSL molecules were added to each mutant culture individually or together at the final concentration 1 μM. The media were incubated at 37 ℃ for 24 hours.

In order to confirm the function of AHL production in mutants and wild type, the modified AHL assay (Kim et al. 2004) was implemented as below: Grow bacterial strains overnight in LB broth at 37 ℃; centrifuge 2 ml bacterial culture for 1 minute, and keep the supernatant; mix the supernatant with 2 ml ethyl acetate by vortexing for 30 second; incubate the samples in room temperature for 5 minutes and take the upper layer to a new tube; air-dry the tubes and dissolve the dried contents (AHL) in 20 μl water; streak the AHL indicator strain *Chromabacterium violaceum* CV026 to LB agar plate, and spot the 20 μl solution to the top of the indicator; LB plates were incubated at 30 ℃ for 48 hours.

2.8 Quantification and Monitoring of Bacteria Growth

To quantify the bacterial cell used in these tests, 12.5 µl and 3 µl overnight cultures were inoculated onto ~ 12.5 ml LB agar and into 3 ml LB broth. The media were incubated at 37 ℃ for 24 hours. The bacterial cells were scraped from agar or precipitated from broth and dissolved into fresh LB broth. The Biomate3 spectrometer was used to measure the optical density (OD) of bacterial cells at 600nm (The bacterial suspensions which had OD values more than 0.6 were diluted and measured again). The absorbance of extracted toxoflavin methanol solution was measured at 393 nm.

2.9 Extraction and Quantification of Toxoflavin from Mutants and Wild Type

Chloroform was used to extract toxoflavin from the bacterial supernatant in this study following the reported protocol with some modifications (Kim et al. 2004). For the bacteria growing in LB broth: spin down 1 ml bacterial culture for 1 minute, and then take the supernatant to a new microcentrifuge tube; mix the supernatant with 1 ml chloroform by vortexing for 30 seconds; incubate the samples in room temperature for 5 minutes and transfer the lower layer to a new microcentrifuge tube; air-dry the tubes and dissolve the dried compounds (toxoflavin) in 1 ml 80% methanol. For the bacteria growing on the LB agar, the extraction was slightly different. After the bacterial cells were scraped from the surface of the media, the agar which had the diffused toxoflavin were weighted and cut into small pieces. Then the agar was mixed with 1:1 (weight : volume) chloroform, incubated at room temperature for about 30 minutes or until the chloroform absorbed maximum amount of toxoflavin, and filtrated using funnel; collect the liquid dripping through the funnel. The rest was the same with the toxoflavin extraction from broth supernatant.

2.10 Onion Virulence Test of the Mutants

Modified onion tissue maceration model (Jacobs et al. 2008) was applied to the relative virulence assay. The outer thin scales of white onion were removed with a sterile razor and the fleshy scales were cut into average pieces (~2 X 4 cm). 48 hours old bacterial culture (grown on LB agar after streaking inoculation) was adjusted to 10^8 cfu using 10 mM $MgCl_2$, and 2 µl suspension was inoculated into the inner bulb scale where wounded by 1~20 µl micropipette tip. The inoculated onion tissues were incubated in a moist chamber at 30 ℃ for 72 hours. The virulence level of different strains was indicated by the maceration area.

2.11 Observation of Production of Virulence Factors on Different Media

Toxoflavin production was indicated by the yellow color of the culture growing on LB and KB agar. Putative virulence factor EPS was indicated by the white and creamy layer on the culture growing on KB and CPG agar, or the cell surface binding ability on the Congo red medium (CRM) (Chung et al. 2003). Lipase was indicated by the opaque and iridescent halo around the bacterial colony growing in LB-Tween 20 agar. Motility of the bacteria were shown on the SWA and SWI agars (Kim et al. 2007).

Chapter 3. Results

3.1 The *tofI* Disruptive Mutants Showed Unstable Phenotypes

The *tofI* disruptive mutants LSUPB214 and LSUPB220 were created as described previously. The insertion of entry vector was confirmed by PCR using primers TofI(H)F and TofR(H)R (Appendix) for both mutants and wild type genomic DNAs (Fig. 1). PCR products were obtained only from wild type DNA samples (Fig. 1 lane 1 and 9) and none from both mutant DNAs (Fig. 1 lane 2, 3, 4 for LSUPB214 and lane 6, 7, 8 for LSUPB 220). The mutant LSUPB214 completely lost the ability to produce toxoflavin (Fig. 1) grown either on LB agar or in LB broth. The mutant LSUPB220 produced no toxoflavin in LB broth, but it did produce, at reduced level, toxoflavin as compared to the wild type when they grew on LB agar (Fig. 2) plate. Surprisingly, the phenotypes of LSUPB220 in solid and liquid media were different. With the supplement of C8-HSL, LSUPB220 produced toxoflavin, but LSUPB214 still failed to produce toxoflavin (Fig. 3) in LB broth. C6-HSL could not complement any of the mutants' toxoflavin production (Fig. 3). The fact that disruptive mutants inserted by similar vectors produced different phenotypes implied the instability of disruptive mutation and the necessity for an alternative mutation strategy.

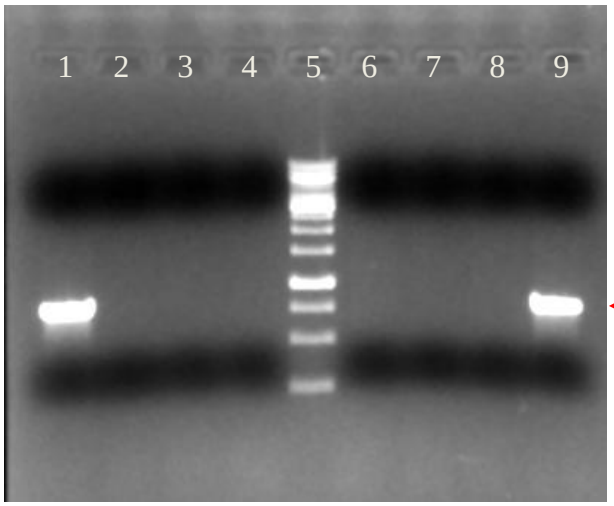


Figure. 1. Confirmation PCR for *tofI* disruptive mutants.

PCR product bands with expected size 800 *bp* amplified and shown on the agarose gel. Lane 1 and 9: PCR products from wild type DNA; Lane 2, 3, 4: no PCR products amplified from DNAs of representative LSUPB214 replicates; Lane 6, 7, and 8: no PCR products amplified from DNAs of LSUPB220 replicates; Lane 5: Generuler^{TM} DNA ladder.

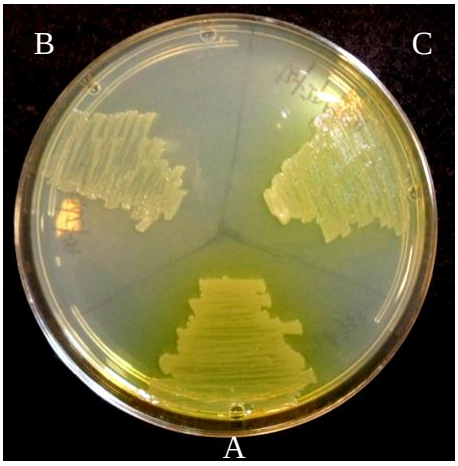


Figure. 2. The toxoflaivn production ability of disruptive mutants

Wild type 336gr-1 (A) produced toxoflavin diffusing to the surrounding area in the agar. LSUPB 214 (*tofI*::Pknock-Km) did not produce any visible toxoflvin (B). LSUPB220 (*tofI*:: Pknock-Gm) produced reduced amount of toxoflavin than the wild type strain (C). Two disruptive *tofI* mutants produced different levels of toxoflavin production in solid media.

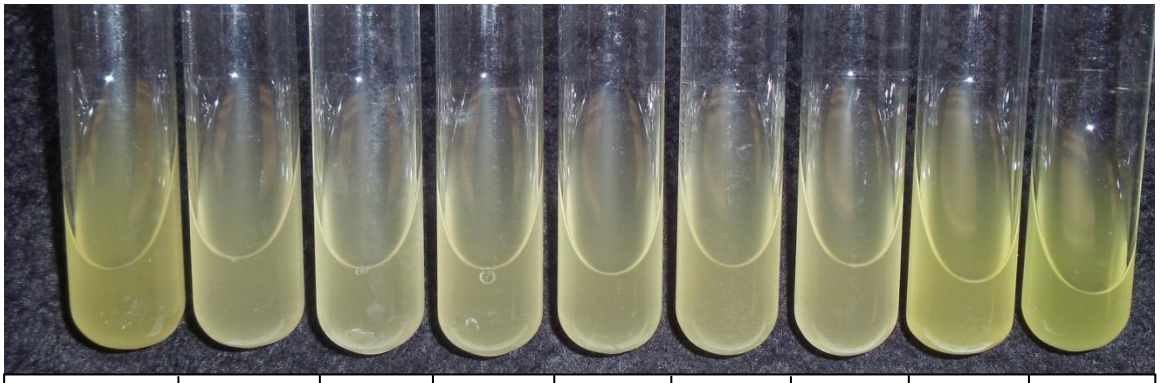


Fig. 3. The complementary test of C6- and C8-HSL to *tofI* disruptive mutants.

Both of the two mutants lost their full toxoflavin producing abilities in LB broth. C6-HSL could not complement the toxoflavin production; C8-HSL could recover the toxoflavin production LSUPB220, but not LSUPB 214. C6- and C8-HSL signals added together to LB broth did not make phenotypic change compared to C8-HSL added only. The 3 ml inoculated samples were (from left to right): 336gr-1 wild type strain in LB, LSUPB214 in LB, LSUPB214 in L6, LSUPB214 in L8, LSUPB214 in L68, LSUPB220 in LB, LSUPB220 in L6, LSUPB220 in L8, LSUPB220 in L68.

3.2 The Development of A Novel Deletion Mutation Tool: pKKSacB Vector

The 2.7 *kb sacB* DNA fragment was sub-cloned into the pKNOCK-Km suicide vector as shown in the diagram below. The *sacB* gene encodes levansucrase, which is able to make the bacterial cells sensitive to sucrose (Ried and Collmer 1987). When *sacB* expresses in *B. glumae*, the bacterial cell will not survive in 10% sucrose. To increase the accuracy of the mutants' screening, 30% sucrose was applied to the selective medium (wild type strains can grow in 30% sucrose medium). It was proved that the *B. glumae* mutant with pKKSacB vector integrated in its genome could not grow on the sucrose medium. But, after the secondary recombination happened between the vector and homologous region, the bacterial cells were recovered and able to grow on the sucrose medium.

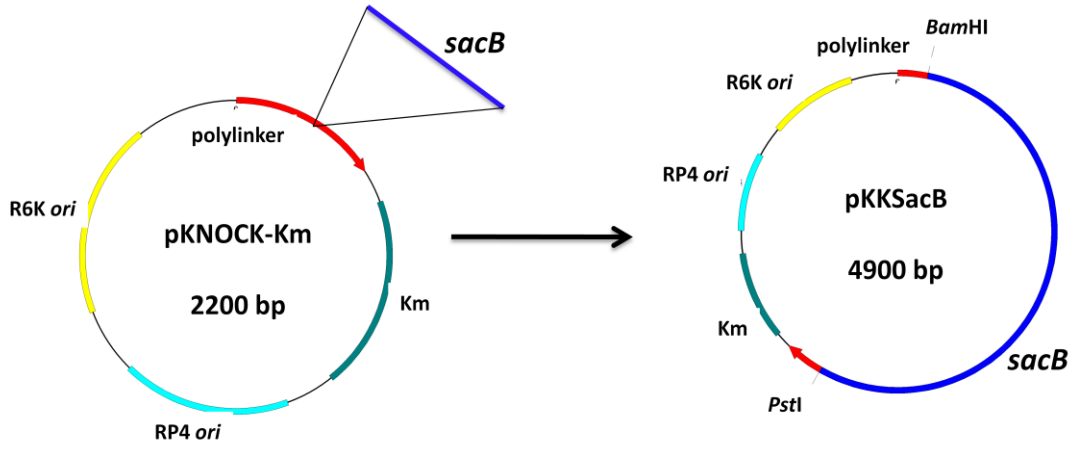


Fig. 4. Construction of the pBBSacB vector.

The *sacB* gene was inserted into the pKNOCK-Km vector at *Bam*HI and *Pst*I sites. pKKSacB has Km gene, RP4 and R6K origins, which give this vector Km resistence, conjugative and suicide properties.

3.3 Construction of Δ*tofI*, Δ*tofI/tofR/orf1*, and Δ*tofR* Deletion Mutants

The Δ*tofI* (LSUPB145), Δ*tofI/tofR/orf1* (LSUPB139), and Δ*tofR* (LSUPB169) mutants were created using the pKKSacB deletion mutation system. The mutant with first recombination occurred in the flanking homologous region had the suicide vector integrated in its genome and was selected from the Km containing plate. The selected primary mutant also had sucrose sensitivity encoded by *sacB* gene. During the next incubation time, the mutant with secondary crossover occurred in another flanking region removed the suicide vector and became insensitive to sugar. Then the double crossover mutant, which was also the deletion mutant, was selected from the sucrose plate. The deletion mutants LSUPB145, LSUPB139, and LSUPB169 were confirmed by PCR with primers corresponding to the adjacent region of the deleted genes (Fig. 5). For every mutant, the size of PCR products from it was equal to the size of PCR products amplified from pKKSacB harboring deletion construct, and the difference between the PCR product from wild type and mutant was the size of the deleted genes.

After both *tofI* and *tofR* and their middle region deleted from the genome, the mutant LSUPB139 lost the toxoflavin production ability on KB and LB media (Fig. 6 and 7), which indicated the importance of quorum sensing genes in toxoflavin production. However, when only *tofI* or *tofR* gene deleted from the genome, the mutants LSUPB145 and LSUPB169 were still able to produce toxoflavin as much as wild type in LB agar (Fig. 6) and KB agar (Fig. 7), which implied the possibility of some other regulatory components. In the 3 ml LB broth, all the mutants did not produce toxoflavin (Fig. 8). The phenotypic difference between the solid and liquid media existed in the quorum sensing deficient mutants (including disruptive mutant LSUPB220), and the reason causing this is still unknown.

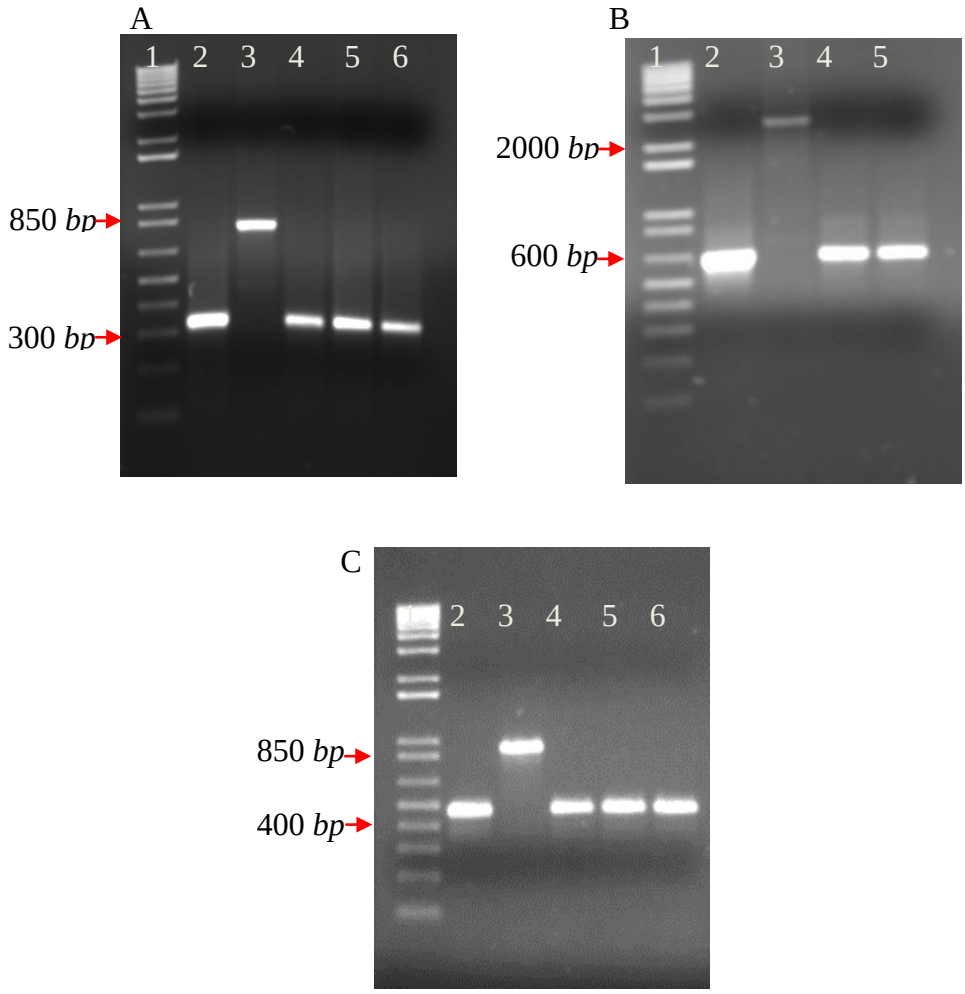


Fig. 5. Confirmation PCR for deletion mutants LSUPB145 (A), LSUPB139 (B), and LSUPB169 (C).

The primers used for *tofI* deletion in LSUPB145 are TofI(H)F and TofI(H)R (Appendix), by which different size of PCR products were amplified from wild type and mutant (A). The expected size of PCR products in wild type was 800 *bp*, while in mutants and positive control vector (pBBSacB with deletion construction) was 318 *bp* (A). The difference 482 *bp* between the two was the DNA fragment length deleted from the target gene locus. Primers TofI(H)F and TofR(H)R (Appendix) were used to confirm the deletion mutation of *tofI*, *tofR*, and the middle region between them (tofI/*tofR/orf1*). In wild type, the PCR products with 2,456*bp* expected size were amplified (B). In mutants and positive control, the PCR products were 586 *bp* in size as expected (B). The difference between the PCR DNA bands attained from wild type and mutant was 1870 *bp*, which equaled to the deleted DNA size deleted from LSUPB139 (B). Using primers TofR(H)F and TofR(H)R (Appendix), DNA bands with expected sizes 911 *bp* and 436 *bp* were produced from wild type and mutant (C). The difference between the PCR products from wild type and LSUPB169 was 475 *bp*, same to the deleted region from *tofR* locus (C). Lane 1: invitrogen 1 kb plus ladder; lane 2: pBBSacB vector harboring flanking region (deletion construct) of target gene; lane 3: wild type strain; lane 4, 5 and 6: the mutants from replicate experiments.

3.4 The AHL Production Assay

As expected, only the wild type produced AHL signal, and the indicator strain *Chromabacterium violaceum* CV026 did not sense AHL from any of the quorum sensing gene deletion mutants (Fig. 9). The indicator used in this study can test the presence of C6-HSL and C8-HSL by producing violacein. But C6-HSL is 7.3 times more sensitive than C8-HSL (McClean et al. 1997). C6-HSL and C8-HSL were synthesized and exported in the same process, so the absence of either of them is convincing enough to prove the dysfunction the AHL producing system. This result is consistent with the toxoflavin production pattern in 3 ml LB broth (Fig. 8).

3.5 AHL Recovery Assay of Different Quorum Sensing Mutants

The mutants and wild type strain were inoculated to LB agar and LB broth media. Different conditions were designed to investigate the toxoflavin productivity of each mutant. C6- or/and C8-HSL were added to confirm missing gene's functional role. Previously, by streaking inoculation, LSUPB145 and LSUPB169 produced toxoflavin in LB agar. But with the spreading inoculation (12.5 µl overnight cultures to 12.5 ml agar) method, none of the quorum sensing deletion mutants produced toxoflavin in solid media (Fig. 10). This phenotypic difference between solid and liquid media might be caused by the original concentration difference of the inoculums, and bacteria may also need high initial density to express the toxin. C8-HSL was able to complement the toxoflavin productivity in LSUPB145, but not LSUPB169 or LSUPB139 (Fig. 10 and Fig. 11), which indicating the important role of *tofR* for activating AHL. The same result pattern was also achieved from the AHL recovery assay in the liquid media (Fig. 11). From the observation by naked eyes, the toxoflavin expression level of LSUPB145 in L8 and L68 was

even higher than the wild type (Fig. 10 and Fig. 11). This experiment also proved the success of deletion mutation strategy in this study.

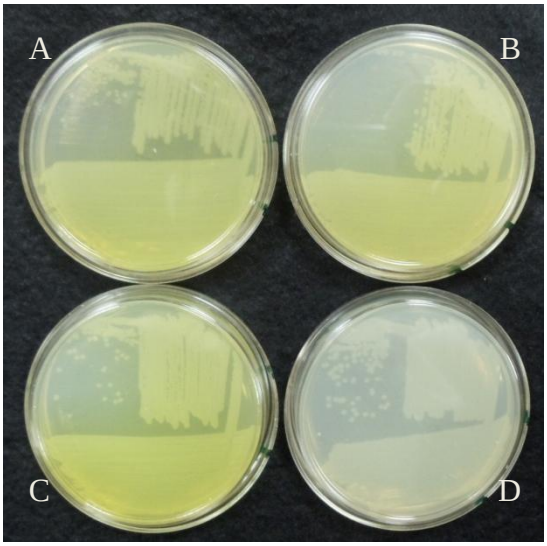


Fig. 6. The toxin producing phenotypes of wild type strain 336gr-1 (A), LSUPB145 (B), LSUPB169 (C) and LSUPB139 (D) in KB plates, 48 hours after streaking inoculation.

LSUPB145 and LSUPB169 could produce yellow toxoflavin as well as wild type. LSUPB139 completely lost the toxoflavin producing ability.

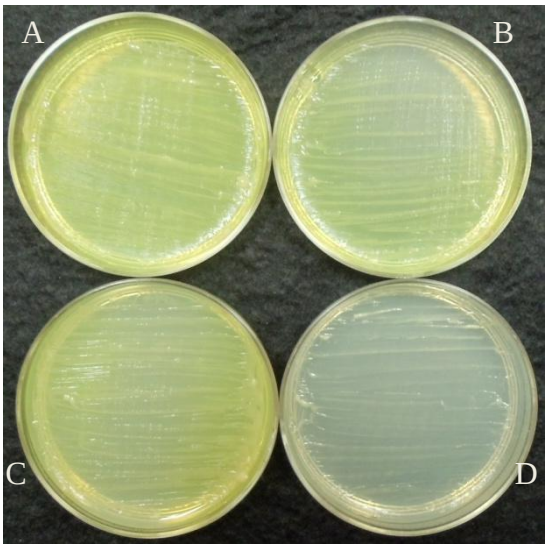


Fig. 7. The toxoflavin expression of wild type and mutants strains in LB agar, 48 hours after streaking incubation.

336gr-1 (A), LSUPB145 (B), LSUPB169 (C) had similar level of toxoflavin expression. LSUPB139 (D) completely lost the toxoflavin producing ability. The result pattern was same with the one observed from KB agar.

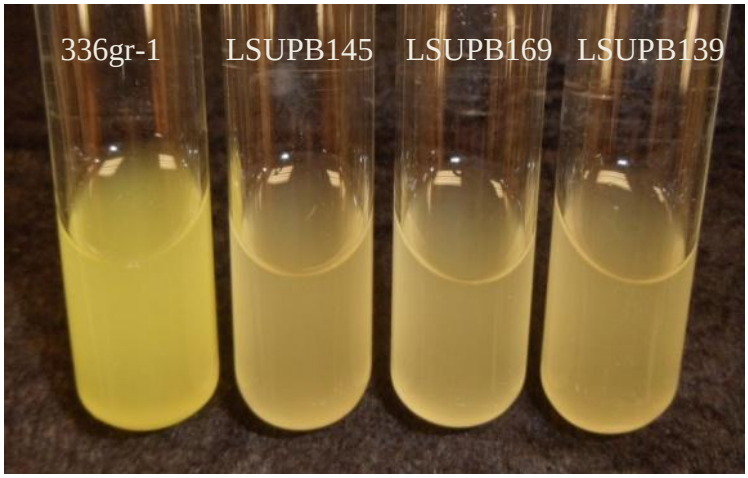


Fig. 8. The toxoflavin production in wild type and quorum sensing deficient mutants in LB broth.

The toxoflavin produced by wild type made the broth culture yellow, but the ones with mutants growing only maintained the original color of LB broth.

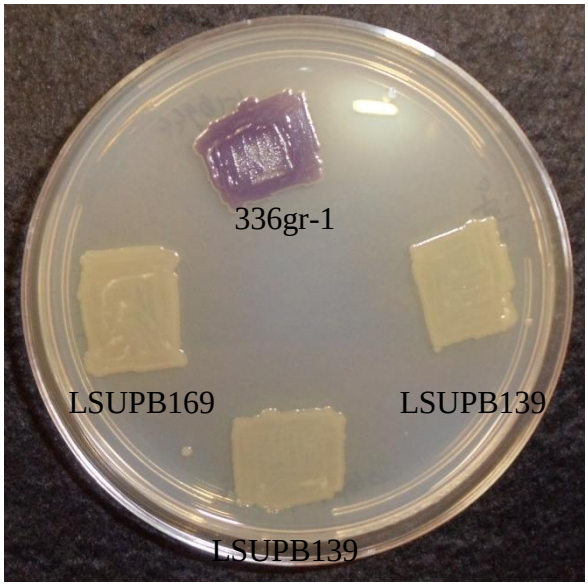


Fig. 9. The AHL productivity of quorum sensing mutants and wild type.
The AHL extracted from 336gr-1 wild type liquid culture induced the violacein expression in *Chromabacterium violaceum* CV026-a quorum sensing and violacein defective mutant. No AHL signal was tested from the mutants' bacterial supernatant by indicator strains.

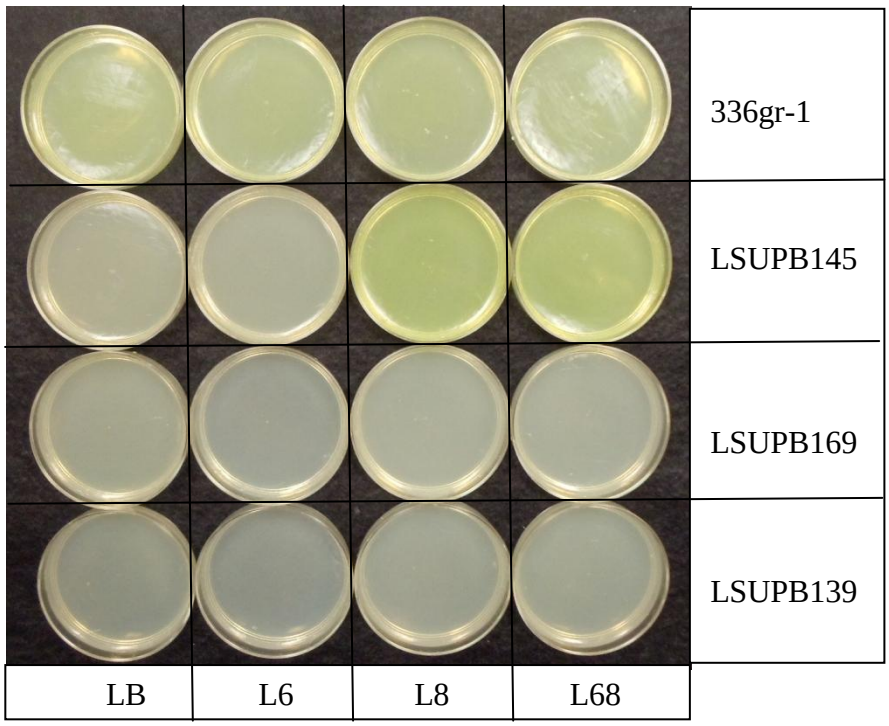


Fig. 10. AHL recovery and toxoflavin expression test for the mutants.

The toxoflavin diffused to agar plate from AHL recovery assay. Picture was taken 48 hours after the bacteria inoculated to the agar plates by spreading. Culture scraped off the surface of the media. Wild type and mutants were treated with AHL signal and compared to the blank control (LB only). LSUPB145 was complemented by C8-HSL, and expressed more toxoflavin than the wild type. LSUPB169 and LSUPB139 could not be recovered by adding artificial AHL signal. C6-HSL failed to complement the toxoflavin production in all the mutants.

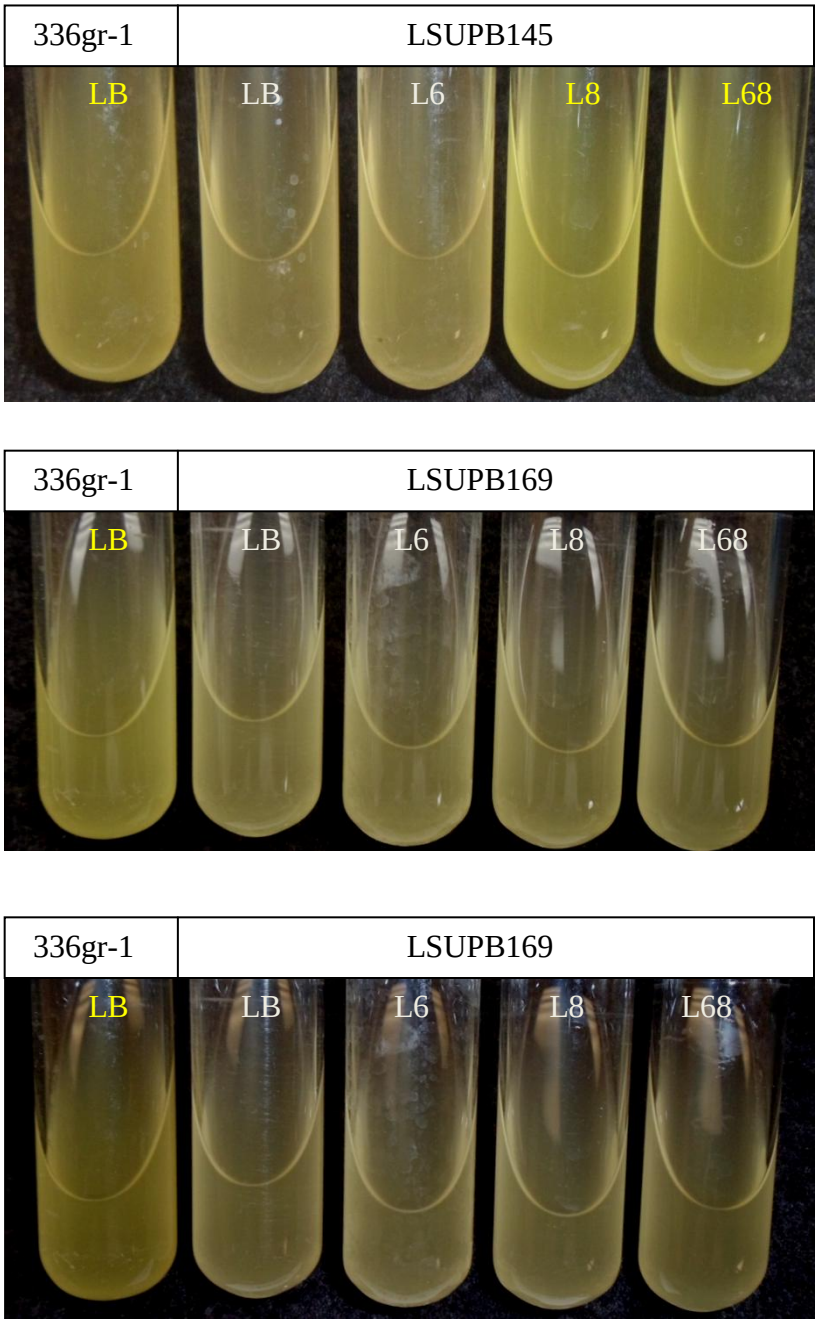


Fig. 11. The toxoflavin expression of the quorum sensing deficient mutants in LB broth media.

This result was corresponding with AHL recovery plate tests. The toxoflavin productivity in LSUPB145 was complemented by 1 μM C8-HSL. As also shown in solid media, the phenotypes of the strains were almost the same in L8 and L68 media.

3.6 Quantification of Bacterial Growth and Toxoflavin Production

To evaluate the recovery effect AHL had on each mutant relative to wild type, the growth and toxoflavin expression of the mutants under different AHL treatments were examined as stated below: 1/1000 (vol of inoculum/vol of medium) overnight culture was inoculated to solid (spreading) and liquid media, and the measurement was performed after 24 hours incubation at 37 ℃. As described above, the toxoflavin and bacterial density were quantified by measuring OD values. The mean of measurements for each strain in defined conditions from three replicates were illustrated in graph (Fig. 12 and Fig. 13). Comparisons of growth between mutants and wild type revealed that the bacterial densities of the mutants were lower than wild type in both solid and liquid media, and the difference was greater in solid media (Fig. 12). Only LSUPB145 responded to C8-HSL in a significant level, and the OD value of its bacterial suspension was higher when C8-HSL was added (Fig. 12). The AHLs did not effect the wild type's growth in both media types, and the added signals also did not accelerate the growths of LSUPB169 nor LSUPB139 (Fig. 12). C6-HSL appeared to slightly depress the toxoflavin production in wild type (Fig. 13). In Fig. 13, mutants barely produced toxoflavin. C8-HSL at 1 μM concentration was able to increase the toxoflavin expression to a level higher than wild type (Fig. 13). For LSUPB145, AHLs' recovery effect on toxoflavin production was greater than on bacterial density (Fig. 12 and Fig. 13). Considering the fact that both bacterial concentration and toxoflavin of LSUPB145 increased with the presence of C8-HSL, there might be some positive relationship between bacterial density and toxoflavin expression. The artificial AHL added in LSUPB145 tended to prevent the bacteria from growing to stationary phase (data not shown), and the increasing bacterial concentration might have induced the production of more toxoflavin, which could be the reason why LSUPB145 produced more toxoflavin than the wild type.

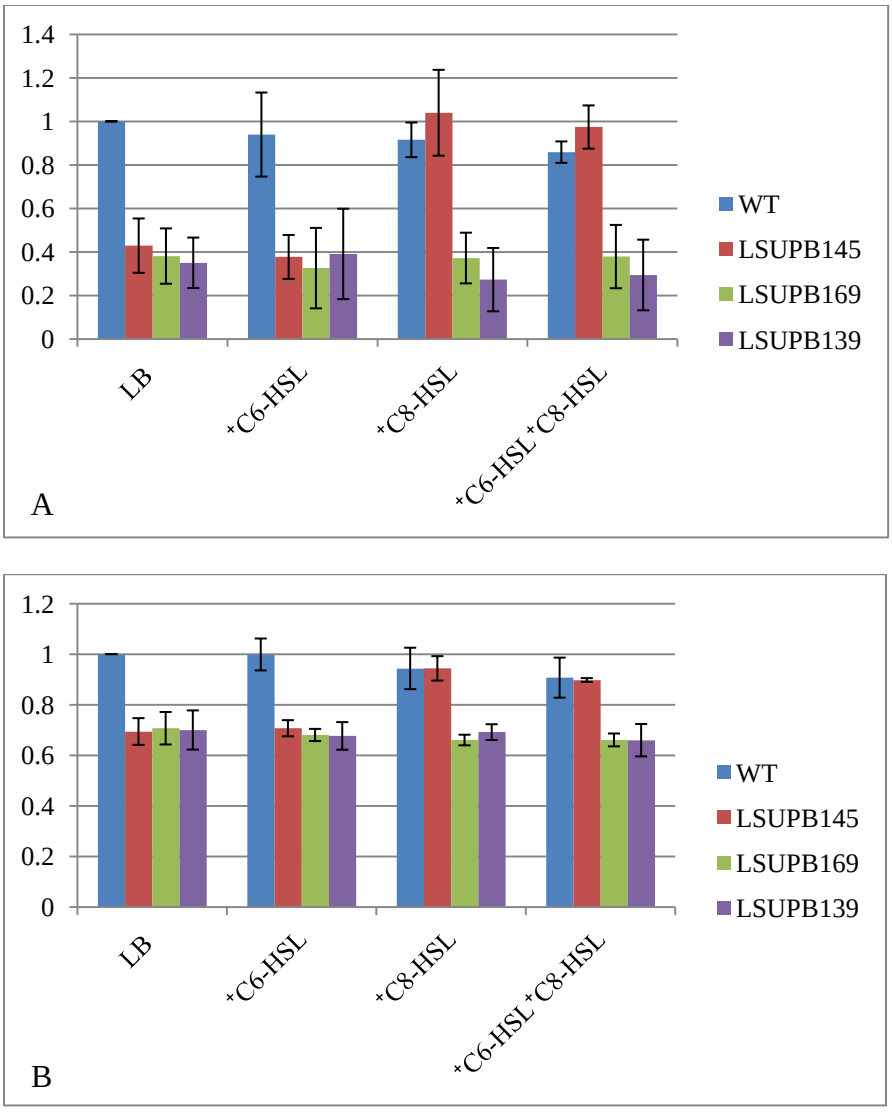


Fig. 12. The relative quantity of individual mutant's bacterial density compared to wild type in solid (A) and liquid media (B).

Three deletion mutants and wild type strains were grown on the LB agar and broth media with different treatments for 24 hours. OD values of bacterial cells were measured at 600 nm wavelength. The parameters from wild type were used as the control (value = 1). The relative data from the mutants were adjusted and the ratio values were recorded and analyzed. The means and SDs were from triplicate experiments. The concentration for the AHL used in all the treatments was 1 μM.

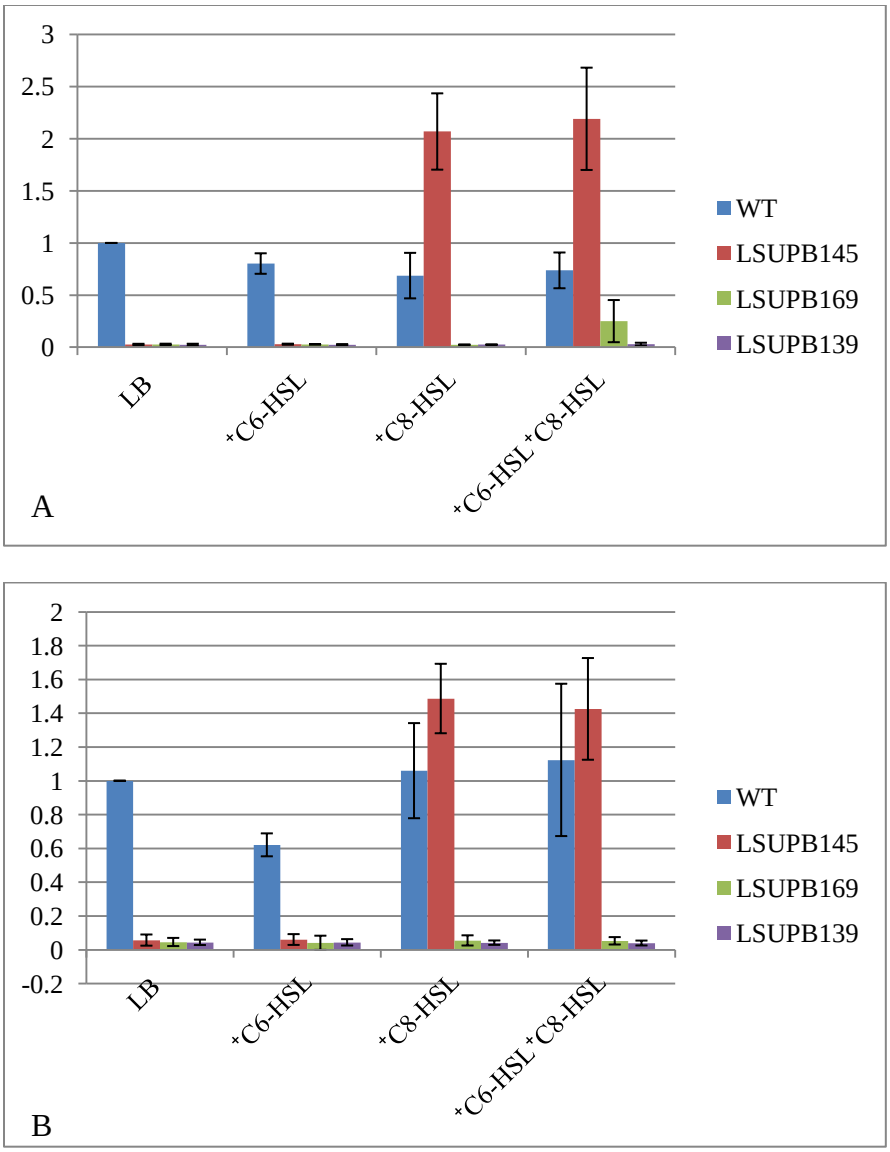


Fig. 13. The relative quantity of toxoflavin expression in quorum sensing mutants compared to wild type in solid (A) and liquid media (B).

The toxoflavin were extracted from the toxin diffused agar and bacterial supernatant after 24 hours incubation. OD values of the toxoflavin in 80% methanol were measured at 393 nm wavelength. The value from wild type was fixed as 1, and the rest of the data were adjusted and analyzed. The means and SDs were from triplicate experiments. The concentrations for the AHL used in all the treatments were 1 μM.

3.7 The Δ*tofI* and Δ*tofR* Mutants Still Pertained Virulence on Onion

To collect the initial data about the virulence of quorum sensing mutants, onion assay was applied to individual derivative mutant strains (Fig. 14). The maceration area was used to evaluate the level of virulence. It turned out that deletion of *tofI* and *tofR* genes did not make *B. glumae* avirulent. In addition, in some replicates for onion assay, LSUPB145 or LSUPB169 even generated bigger maceration areas than wild type. On the other hand, the deletion of *tofI/tofR/orf1* made the mutants no longer pathogenic to onion.

3.8 The Quorum Sensing Mutants Still Maintained Lipase Productivity

The 2 μl overnight bacterial cultures adjusted to 10^9 cfu were dropped in the center of lipase media. After 72 hours incubation at 37 ℃, LSUPB145, LSUPB169 and LSUPB139 produced similar amount of lipase compared to wild type. The sheen halos were observed around the spot culture in LB agar plates. The deletion of quorum sensing genes did not affect lipase production, indicating that another functional regulating system of lipase still existed in the genome.

3.9 The Quorum Sensing Genes Related to EPS Production

To distinguish the EPS-producing variants, colonial morphology on CPG and KB media were used. Wild type appeared white and mucoid surface on KB and CPG media, and the phenotypes were more obvious on CPG medium. The quorum sensing mutants did not show EPS-producing morphology (Fig. 17B, C, and D). EPS also makes the bacteria harder to uptake and bind to Congo red dye, which gives the bacterial colony pink color appearance. On the contrary, the bacteria which do not produce EPS have red color appearance. As shown in Fig. 17, pink color indicated the production of EPS in 336gr-1, whereas red color on the colonial surface

indicated the reduced EPS produced by mutants. Evidently, the quorum sensing genes *tofI* and *tofR* probably involved in the production of EPS.

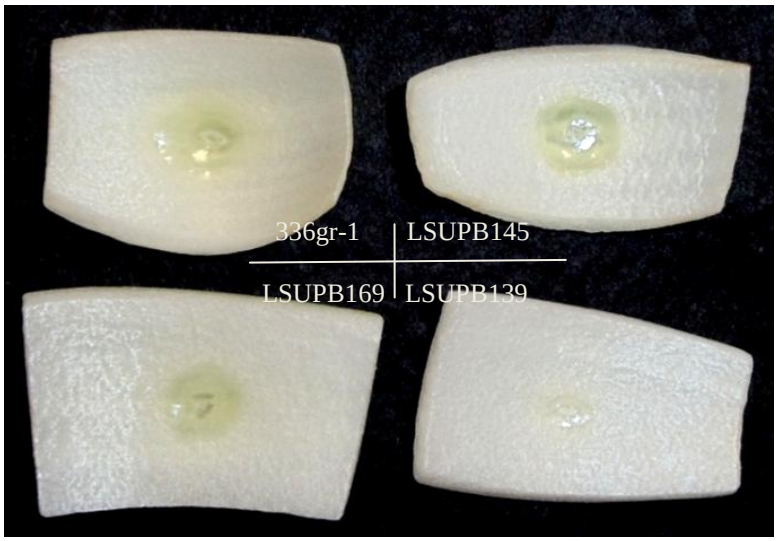


Fig. 14. The onion virulence test and the maceration caused by *B. gluame.*

LSUPB145 and LSUPB169 caused similar type of maceration with wild type. The onion started rotting from the inoculated spot and the macerated tissue appeared yellow toxin under the surface. In contrast, LSUPB139 did not show any symptoms on onion, which was probably due to the loss of major virulence factors.

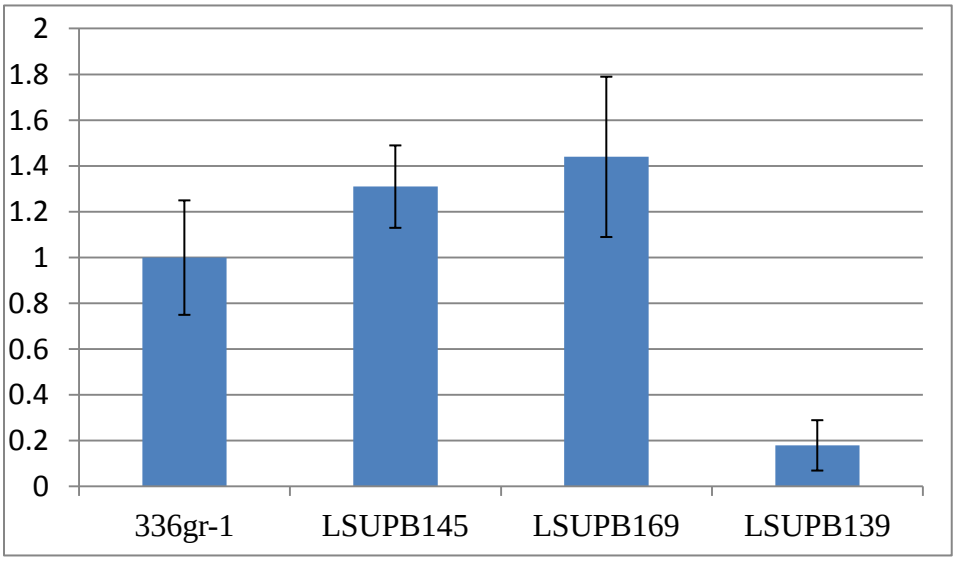


Fig. 15. Relative virulence of quorum sensing mutants compared to wild type on onion.

The long (a) and short (b) diameter of the maceration region caused by each strain was measured, and the area was calculated by following the formula: $A = \pi(\frac{a}{2} + \frac{b}{2})^2$. The columns indicated the mean of the relative maceration area caused by each strain with wild type.

3.10 The Quorum Sensing Defective Mutants Still Had Motility Property

The swimming and swarming activities of the mutants and wild type strains were examined at 30 ℃ and 37 ℃ respectively. All the tested bacteria showed swimming phenotypes, meanwhile all the variants except LSUPB139 performed swarming (Fig. 18). Based on the observation from the motility assay in this study, quorum sensing system was not critical for swimming. The quorum sensing regulator is probably important for swarming of *B. glumae*. The levels of the swimming and swarming were not rated yet (in the plan of future work), and the pictures did not accurately represent all replicates for motility tests.

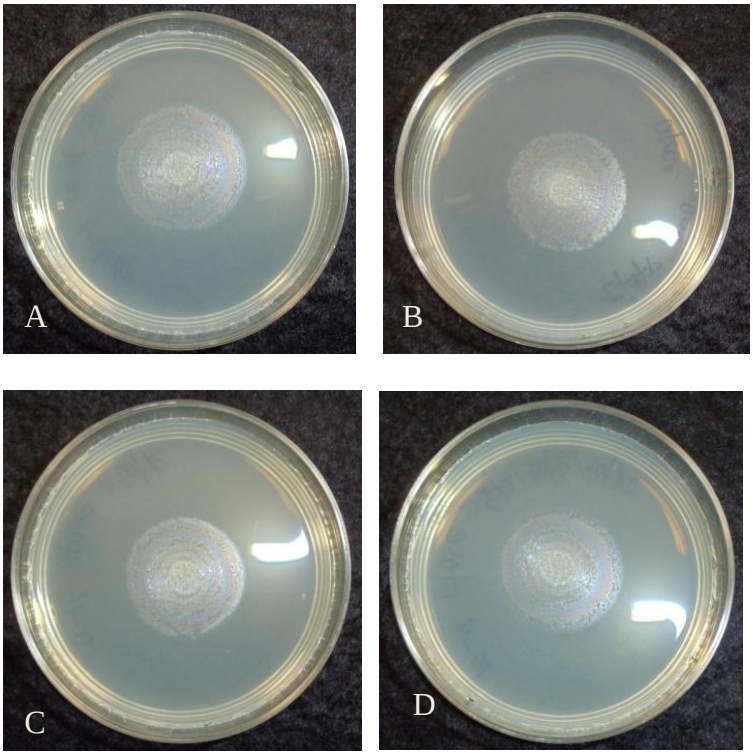


Fig. 16. The quorum sensing defective mutants did not loose lipase productivity.

The halo around bacterial colony indicated the activity and production of the lipase. Wild type (A), LSUPB145 (B), LSUPB169 (C) and LSUPB139 (D) all generated sheen halo around them on the lipase media. Yet there were no significant difference between the halo sizes produced by wild type and mutants.

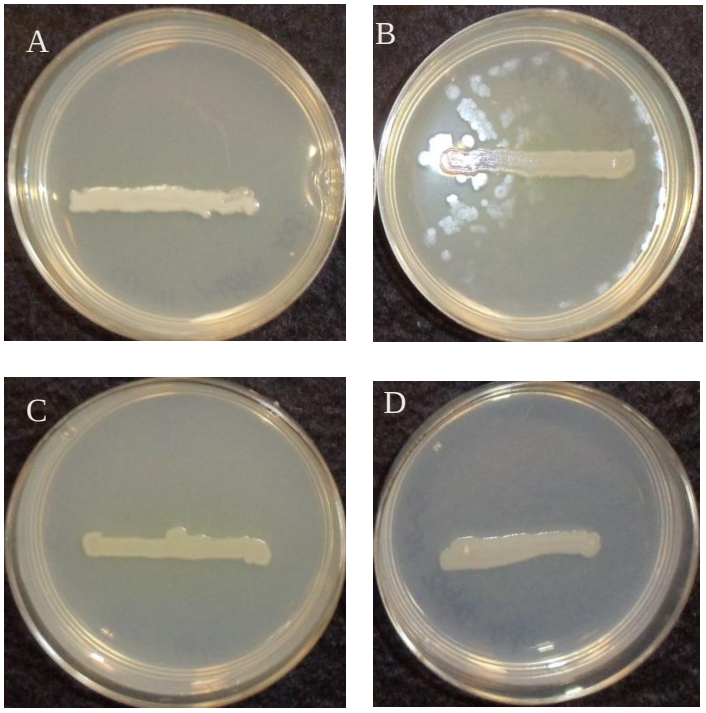
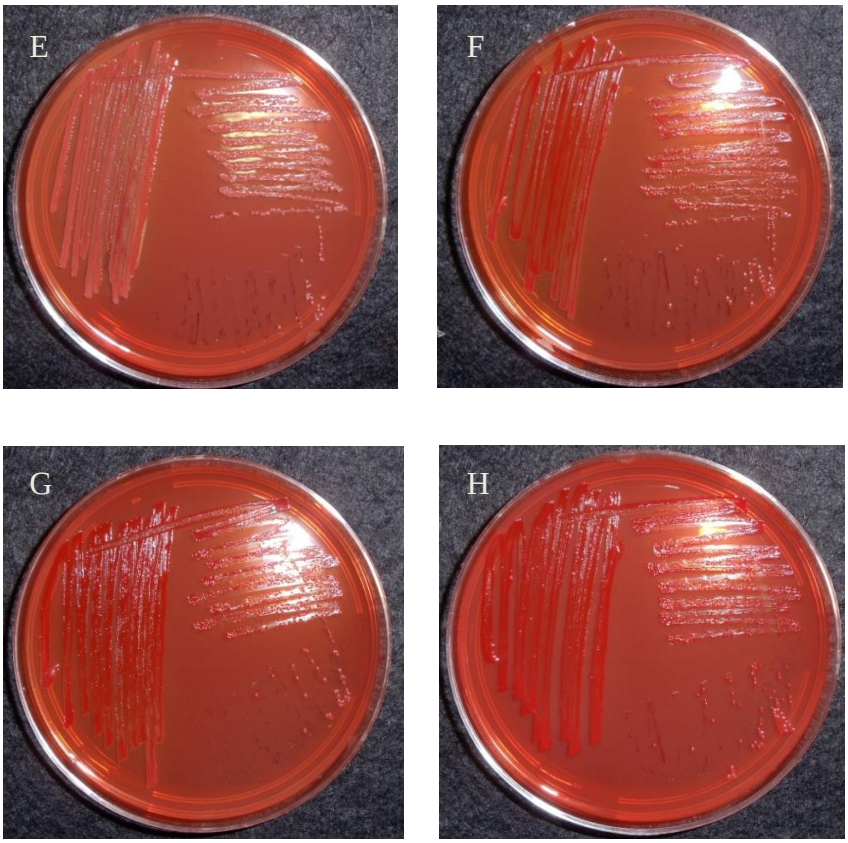


Fig. 17. The colonial EPS morphology of the wild type and quorum sensing mutants on CPG and CRM plate.

EPS produced by wild type was indicated by the white and mucoid surface (A) on CPG media, while the mutants LSUPB145 (B), LSUPB169 (C), and LSUPB139 (D) had drier surfaces. The Congo red binding abilities of the bacteria were indicated by the color of the colonial surfaces. 336gr-1 produced EPS and bound fewer dyes, which gave the colony a pink appearance (E). By comparison, all the mutants bound more dyes and had a red colonial surface (F, G and H). F: LSUPB145; G: LSUPB169; H: LSUPB139.



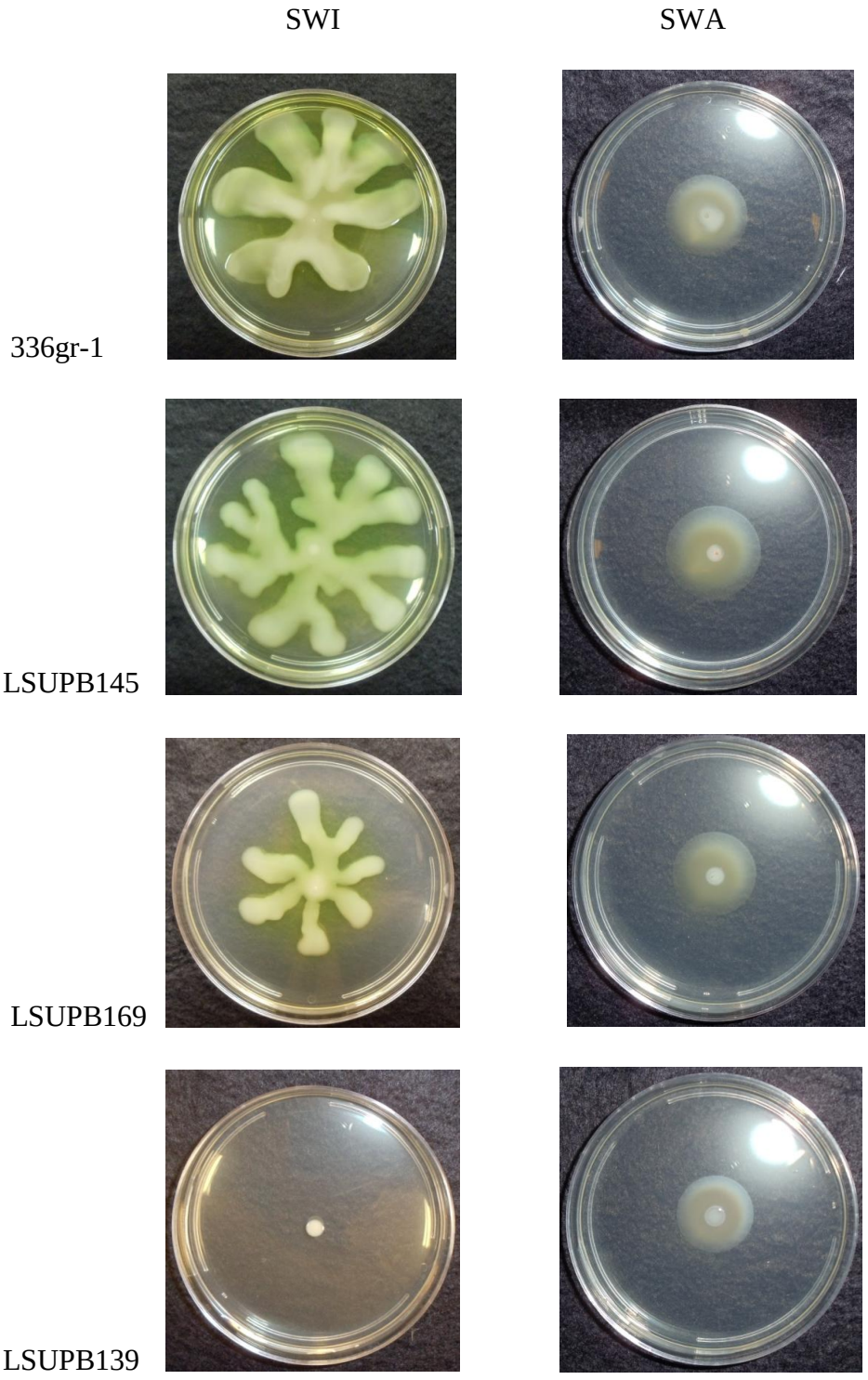


Fig. 18. The motility test of the studied quorum sensing derivatives.

Both wild type and mutants showed swimming activites, but not all of them had swarming type motility.

3.11 Development of A New Deletion Mutant Δ*tofI*/Δ*tofR* (LSUPB201)

To investigate the function of ORF *orf1*, which is located in the middle region (upstream) of *tofI* and *tofR*, the *tofI* and *tofR* genes were deleted sequentially and left *orf1* in the genome. As mentioned previously, the *tofI* gene was deleted first after double recombination between wild type genome and pBBSacBΔ*tofI* entry vector. Later, in the Δ*tofI* background, *tofR* gene was deleted by introducing pBBSacBΔ*tofR* deletion construct to the transformant. The confirmation PCR proved the successful deletion of both *tofI* and *tofR* genes from the genome of mutant LSUPB201 (Fig. 18).

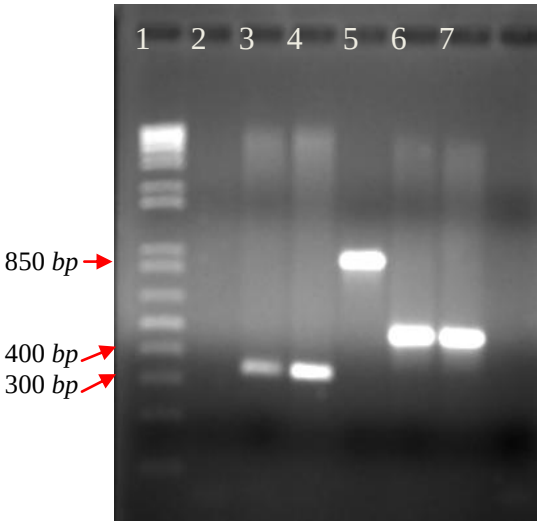


Fig. 18. Confirmation PCR for *tofI* and *tofR* deletion in LSUPB201.

PCR products from *tofI* and *tofR* deleted DNA regions were shown at the expected size, which equaled to the lengths of DNAs amplified from LSUPB145 (Fig. 5A) and LSUPB169 (Fig. 5C). Lane 1: invitrogen 1 kb plus ladder; lane 2: PCR of wild type strain using primers TofI(H)F and TofI(H)R (Appendix A) (PCR did not work probably due to the change of the buffer, but the new designed programs are being attempted); lane 3 and 4: PCR of LSUPB201 replicate mutants using primers TofI(H)F and TofI(H)R; lane 5: PCR of wild type strain using primers TofR(H)F and TofR(H)R (Appendix A); lane 6 and 7: LSUPB201 replicate mutants using TofR(H)F and TofR(H)R.

3.12 The Phenotypic Characteristics of the LSUPB201

With *tofI* and *tofR* genes deleted from the quorum sensing system, LSUPB201was still able to produce toxoflavin (Fig. 19A) in the LB agar after streaking inoculation and AHL (Fig. 19C). No toxoflavin was produced in 3 ml LB in test tube, and this phenotype could not be complemented by adding AHL signal (Fig. 19B). Compared with the toxin null mutant LSUPB139, the left *orf1* gene in LSUPB201 might complement the regulation of toxoflavin production. LSUPB201 is still virulent to onion, and caused maceration in a level similar to wild

type (Fig. 19D). This mutant showed lipase (Fig. 19E), swimming (Fig. 19I) and swarming activity (Fig. 19H). Yet, LSUPB201 did not produce visible EPS (Fig. 19F), and the culture appeared red surface in CRM plate (Fig. 19G).

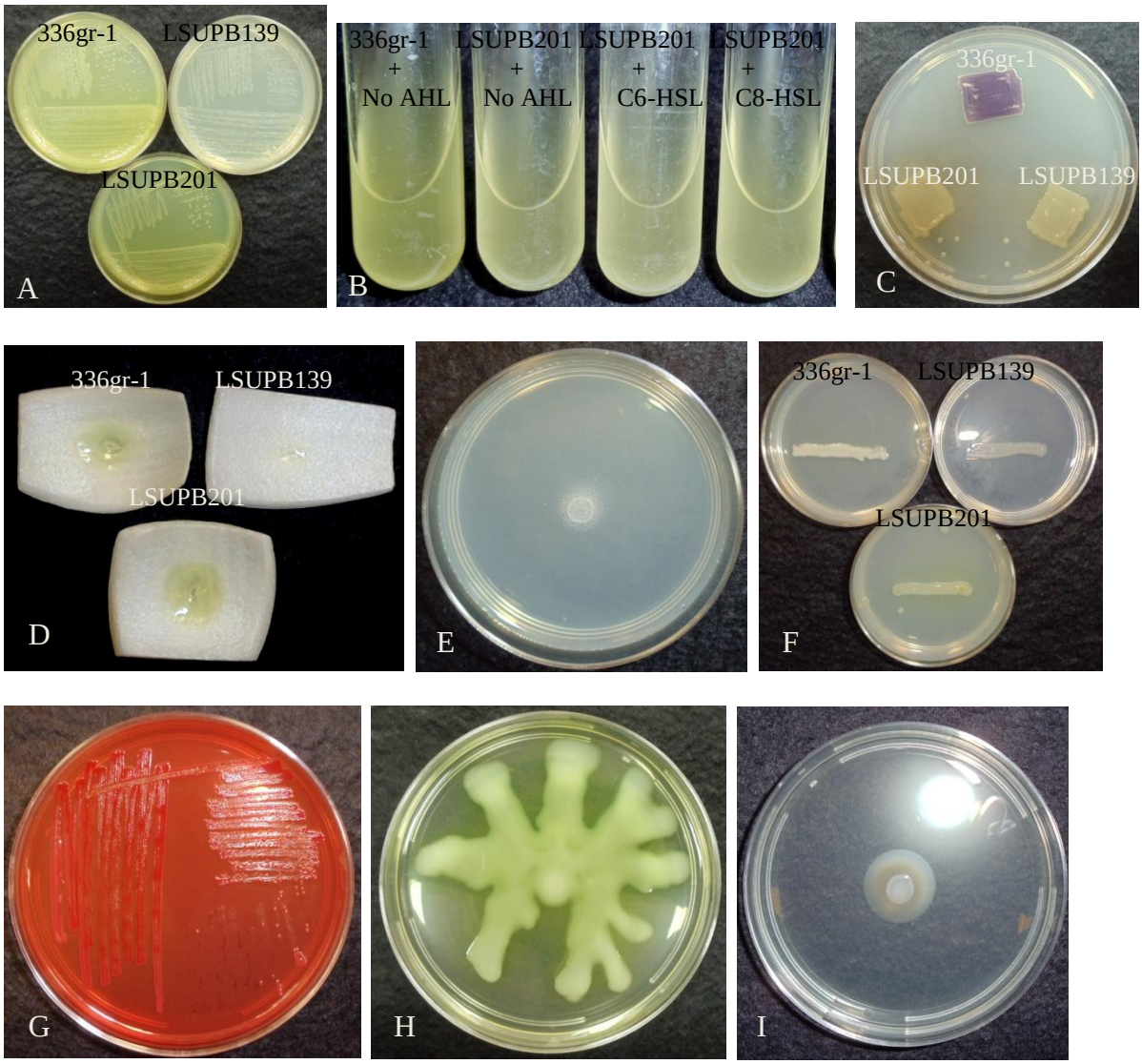


Fig. 19. The phenotypic tests for mutant LSUPB201.

This mutant produced yellow toxoflavin in LB agar (A). But it did not produce toxoflavin in LB broth in test tubes (B), and could not be recovered by adding AHL signal (B). This mutant could cause maceration in a similar level with wild type (C). Lipase productivity was also observed on lipase medium (D). No EPS was shown on the bacterial surface in the CPG medium (E); same reason, the bacteria could bind on Congo red dye well and appeared red color (F). Swarming (G) and swimming (H) were shown on 0.4 % agar.

Chapter 4. Discussion

4.1 Development of A Novel Mutagenesis Strategy

A novel mutagenesis system, pKKSacB counter selection system, for quorum sensing genes was developed. At first, a *tofI* disruptive mutant was obtained by single crossover recombination between the pKNOCK suicide vector containing the internal *tofI* gene homologous fragment and the *B. glumae* genome. During the toxoflavin production assay, *tofI*::pKNOCK-Km and *tofI*::pKNOCK-Gm were observed expressing different phenotypes. The potential explanation of this phenomenon could be existence of a functional truncated protein of the target gene or the possible effect of the vector insertion on the downstream negative regulator of the virulence gene. Because of the inconsistence of the disruptive single crossover mutant, pKKSacB vector, which used sucrose as counter selective marker, was built up to achieve deletion mutant. pKKSacB suicide vector has been used to make all the quorum sensing deficient deletion mutants in this study, and the genetic and phenotypic confirmation demonstrated the success of the pKKSacB mutation system. In addition, compared to another disruptive mutant, LSUPB220 (*tofI*:: pKNOCK-Gm) was more similar to the *tofI* deletion mutant LSUPB145. Therefore, pKNOCK-Gm suicide vector would be a better choice to mutate the target gene than the pKNOCK-Km suicide vector.

4.2 Implification of New Quorum Sensing Interacting Systems

The phenotypes of quorum sensing deletion mutants were studied by a series of examinations. In brief, quorum sensing mutants still contained most of the tested phenotypes as well as the wild type. These results were not in agreement with other research groups' published data on strains BGR1 and AU6208 (Devescovi et al. 2007; Kim et al. 2004). The differences may come from the mutation strategy; moreover, genome sequence comparison between BGR1

and 336gr-1 appeared significantly different (Francis and Ham, unpublished data), which could also be the reason why regulation of virulence factors in 336gr-1 was more complicated.

This study confirmed the regulating role of the quorum sensing system on different virulence factors, and revealed the possibility that other independent, unknown regulators interact with the quorum sensing system components (TofI or TofR). *B. glumae* toxoflavin production was not completely lost in the Δ*tofI* (LSUPB145) and Δ*tofR* (LSUPB169) mutants, while it was barely produced in the Δ*tofI*/*tofR*/*orf1* (LSUPB139) mutant. In BGR1, both *tofI* and *tofR* were critical for toxoflavin expression under quorum sensing positive regulation, where the *tofI*::Ω mutant failed to produce both AHL and phytotoxin (Kim et al. 2004). In our study, LSUPB145 and LSUPB169 were still able to express similar amounts of toxoflavin with wild type in agar plate, even though there was no testable AHL signal excreted from those bacterial cultures. But quorum sensing is still important for virulence factor expression, as LSUPB139 consistently failed to produce toxoflavin after all the *tof* components of quorum sensing were deleted. Therefore, it is very likely for *B. glumae* to have another toxin regulator, which was independent of TofI or TofR, but required part of the quorum sensing function.

4.3 Quorum Sensing Mutants Showed Different Phenotypes from Spreading and Streaking Inoculation Methods

On the LB solid medium, the same mutant showed different toxoflavin producing levels after using the streaking and spreading inoculations, whereas the wild type expressed toxoflavin after using both inoculation methods. The disruptive mutant LSUPB220 (*tofI*), the deletion mutant LSUPB145 (*tofI*), and other quorum sensing deficient mutants all produced toxoflavin after being streaked on the agar. Conversely, neither the LSUPB145 nor the LSUPB169 mutant produced yellow toxin on agar plates when the spreading inoculation method was used. In streaking method, more bacterial inoculum was used. Thus, the initial concentration of the

inoculum may be important for toxoflavin expression in these mutants. The quantification of bacterial growth and toxoflavin expression by streaking method will soon be executed. Afterwards, the relationship between inoculum concentration and bacterial behavior will be evaluated. Hopefully, valuable disease development information can be gained from this experiment.

4.4 C8-HSL Can Over-complement the Toxoflavin Production of LSUPB145

This study confirmed the key role of C8-HSL in regulating toxoflavin production, whereas the sensing function of C6-HSL is still not well understood. C6-HSL slightly depressed wild type bacterial growth in the quantification test. In both LB agar and broth media, 1 μM C8-HSL could recover the production of toxoflavin in the *tofI* mutant, to a level even greater than that expressed in wild type isolate. With C8-HSL added to the culture, LSUPB145 not only reached a higher level of toxoflavin expression, but also a higher bacterial concentration compared to the culture with no AHL added. To explain this phenomenon, an experiment to quantify the differences in bacterial growth between LB and L8 media was performed. It was found that additionary C8-HSL tended to delay LSUPB145 from reaching the stationary phase (data not shown). In *B. cepacia*, quorum sensing negatively regulated stationary phase factor RpoS (Aguilar et al. 2003), which was reported not involved in pathogenicity of *B. glumae* (Devescovi et al. 2007). It is possible that more than natural amount of C8-HSL depressed *rpoS* expression, and made the genes' expression stay in late exponential phase pattern, during which toxoflavin expression is highly efficient.

To detect if there were some complementary relationships between C6- and C8-HSL, the samples treated with C6- or C8-HSL were compared with the samples treated with C6- and C8-HSL together. But, results from toxoflavin production and quantification experiments showed no

significant interrelationship between the two AHL signals. The bacteria constantly showed similar phenotypes in L8 and L68 media.

4.5 393 nm Wavelength Was Used to Measure the Absorbance of Toxoflavin

In previous studies, 260 nm was used to measure the OD value of the toxoflavin in methanol solvent (Kim et al. 2004; Karki and Ham, unpublished data), but in this research the spectrometer did not give stable and accurate absorbance with this wavelength. It was revealed from the structural study of toxoflavin that there were two absorption peaks for toxoflavin-258 nm and 393 nm (Jung et al. 2011). Thus 393 nm wavelength was used to quantify the extracted toxoflavin and showed consistent values.

4.6 Toxoflavin and Swarming Motility Were the Major Virulence Factors Identified in these Experiments

In this study, *tofI* and *tofR* mutants showed virulence on onion, which indirectly indicated their virulence on rice. Even though for other strains, the intactness of TofI-TofR regulator was critical for pathogenicity, this result corresponded to the fact that virulence factors still expressed in LSUPB145 and LSUPB169. Toxoflavin and swarming system were the virulence factors that did not express only in LSUPB139. Meanwhile, mutant LSUPB139 did not cause symptoms on onion. Those two results suggested that toxoflavin and swarming motility were the major virulence factors other than lipase or EPS, because lipase expressed in LSUPB139 and EPS did not express in all the quorum sensing mutants.

Even though lipase is possibly not a virulence factor for 336gr-1 to cause panicle blight, the study of this industrial product lipase still has some economic importance. Lipase activities of the quorum sensing mutants were detected on tween20 media. Unlike the published data, which indicated that both the *tofI* and *tofR* genes were required for the normal secretion of lipase

(Devescovi et al. 2007), even LSUPB139 (Δ*tofI*/*tofR*/*orf1*) demonstrated lipase activity. So, the regulator of lipase in 336gr-1 could be different to other strains. In the *Burkholderia* genus, LuxI-LuxR type quorum sensing was proved to have the regulating role of lipase in *B. thailandensis* and *B. cenocepacia* (Aguilar et al. 2003; Lewenza et al. 1999).

EPS was produced by wild type, but not the quorum sensing mutants. EPS also makes Congo red dye harder to bind on bacterial surfaces, thus CRM plates were used to do routine phenotypic tests for EPS and gained distinguishable results. Based on observation, the intact quorum sensing system was necessary for normal EPS expression, which was different with the expressing pattern of toxoflavin, lipase, and motility. Meanwhile, the quorum sensing mutants all performed the maceration reaction on onion, which meant the deficiency of EPS did not affect the virulence level of *B. glumae.*

LSUPB145, LSUPB169 and LSUPB139 all performed swimming motility at 30 ℃, and LSUPB139 lost swarming motility at 37 ℃. For strain BGR1, the *tofI*::Ω mutant was aflagellate at 37 ℃, and lost swarming ability (Kim et al. 2007). But in this study, LSUPB145 and LSUPB169 were still able to perform swarming; only LSUPB139 lost this ability. Analogous to toxoflavin, the quorum sensing component that was required for motility behavior in 336gr-1 still needs to be clarified. As mentioned above, swarming motility should be one of the major virulence factors in 336gr-1, so better understanding of its regulation would contribute to BPB control.

4.7 The Postulation of *orf1* Functional Quorum Sensing Component

According to NCBI sequence information, another opening reading frame (bglu_2g14480) encoding a hypothetical protein exists between *tofI* and *tofR* genes. From the previous results, when *tofI* and *tofR* were deleted from 336gr-1 separately, toxoflavin and

swarming motility were still expressed. But when *tofI/tofR/orf1* all deleted from the genome, LSUPB139 lost those two phenotypes and virulence. It was not clear if it was *orf1* complemented the function of deleted *tofI* or *tofR.* To determine the role of *orf1* in the regulating system, *tofI* and *tofR* genes were deleted from the genome consecutively leaving only *orf1* in the quorum sensing region. Interestingly, Δ*tofI/ΔtofR* mutant (LSUPB201) could express toxoflavin, swarming and virulence. Compared to genome of avirulent mutant LSUPB139, *orf1*, which was kept in LSUPB201, may play essential role in the virulence factors' expressing or be the collaboration key between quorum sensing and some other regulators. To my knowledge, there is no description of the hypothetical protein encoded by the ORF located between *tofI* and *tofR* genes in *B. glumae*. More detailed study about ORF1 will be implemented and this may contribute to understanding the quorum sensing system in *B. glumae.*

4.8 Conclusion and Significance

Summing up, this study created a reliable deletion mutation strategy-pBBSacB counter selection system; provided comparison data about the expression of known and putative virulence factors in 336gr-1 variants; and analyzed their relation with TofI-TofR quorum sensing regulator. Toxoflavin and swarming motility were confirmed to be the major virulence factors, and they were partially regulated by quorum sensing. The putative function of *orf1* gene was pointed out, which may contribute to the expression of major virulence factors. Future work will be focused on the determination of other independent regulators of toxoflavin and motility involved in pathogenicity, and the genetic study of *orf1* using a similar strategy. The study of the regulation of pathogenicity can provide important information for the control of BPB.

This study has some horizontal significance too. As different data were attained from 336gr-1 and other *B. glumae* strains, it will be interesting to know if intra-species evolution

occurred due to the different geographical locations. The supplement of quorum sensing knowledge and genetic manipulation methods in *B. glumae* can also be used for the study of other *Burkholderia* spp. Even though the importance of *Burkholderia* spp for their virulence and biological benefit has been emerging, the health risk from many of them worries people about the widespread use of the bacteria. This worry hampers the study of most of the species by strict regulations. On the other hand, *B. glumae*, as a known plant pathogen, does not have so many biosafety and security problems. Some techniques and the knowledge base for determining the molecular traits of *B. glumae*, developed in this study, may also be applied to other species.

References

Aguilar C, Bertani I, Venturi V (2003) Quorum-sensing system and stationary-phase sigma factor (rpoS) of the onion pathogen Burkholderia cepacia genomovar I type strain, ATCC 25416. Appl. Environ. Microbiol. 69: 1739-1747

Alexeyev MF (1999) The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. Biotechniques 26: 824-828

Atkinson S, Williams P (2009) Quorum sensing and social networking in the microbial world. J. R. Soc. Interface 6: 959-978

Baldwin A, Sokol PA, Parkhill J, Mahenthiralingam E (2004) The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect. Immun. 72: 1537-1547

Bartholdson SJ, Brown AR, Mewburn BR, Clarke DJ, Fry SC, Campopiano DJ, Govan JRW (2008) Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex. Microbiology-Sgm 154: 2513-2521

Boon C, Deng YY, Wang LH, He YW, Xu JL, Fan Y, Pan SQ, Zhang LH (2008) A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. Isme J. 2: 27-36

Chien CC, Chang YC (1987) The susceptibility of rice plants at different growth stages and of 21 commercial rice varieties to *Pseudomonas glumae*. J. Agric. Res. China 36: 302-310

Chun H, Choi O, Goo E, Kim N, Kim H, Kang Y, Kim J, Moon JS, Hwang I (2009) The quorum sensing-dependent gene *kat*G of *Burkholderia glumae* is Important for protection from visible light. J. Bacteriol. 191: 4152-4157

Chung JW, Altman E, Beveridge TJ, Speert DP (2003) Colonial morphology of Burkholderia cepacia complex genomovar III: Implications in exopolysaccharide production, pilus expression, and persistence in the mouse. Infect. Immun. 71: 904-909

Coenye T, Vandamme P (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ. Microbiol. 5: 719-729

Conway BA, Greenberg EP (2002) Quorum-sensing signals and quorum-sensing genes in Burkholderia vietnamiensis. J. Bacteriol. 184: 1187-1191

Conway BAD, Chu KK, Bylund J, Altman E, Speert DP (2004) Production of exopolysaccharide by *Burkholderia cenocepacia* results in altered cell-surface interactions and altered bacterial clearance in mice. J. Infect. Dis. 190: 957-966

Cornelis GR, Van Gijsegem F (2000) Assembly and function of type III secretory systems. Annu. Rev. Microbiol. 54: 735-774

Cottyn B, Cerez MT, VanOutryve MF, Barroga J, Swings J, Mew TW (1996) Bacterial diseases of rice .1. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80: 429-437

de Kievit TR, Iglewski BH (2000) Bacterial quorum sensing in pathogenic relationships. Infect. Immun. 68: 4839-4849

Deng YY, Wu JE, Eberl L, Zhang LH (2010) Structural and functional characterization of diffusible signal factor family quorum-sensing signals produced by members of the *Burkholderia cepacia* complex. Appl. Environ. Microbiol. 76: 4675-4683

Devescovi G, Bigirimana J, Degrassi G, Cabrio L, LiPuma JJ, Kim J, Hwang I, Venturi V (2007) Involvement of a quorum-sensing-regulated lipase secreted by a clinical isolate of *Burkholderia glumae* in severe disease symptoms in rice. Appl. Environ. Microbiol. 73: 4950-4958

Di Cagno R, De Angelis M, Calasso M, Gobbetti M (2011) Proteomics of the bacterial cross-talk by quorum sensing. J. Proteomics 74: 19-34

Duerkop BA, Herman JP, Ulrich RL, Churchill MEA, Greenberg EP (2008) The *Burkholderia mallei* BmaR3-BmaI3 quorum-sensing system produces and responds to N-3-hydroxy-octanoyl homoserine lactone. J. Bacteriol. 190: 5137-5141

Duerkop BA, Ulrich RL, Greenberg EP (2007) Octanoyl-homoserine lactone is the cognate signal for *Burkholderia mallei* BmaR1-BmaI1 quorum sensing. J. Bacteriol. 189: 5034-5040

Eberhard A (1972) Inhibition and actication of bacterial luciferase synthesis. J. Bacteriol. 109: 1101-1105

Eberl L, Riedel K (2011) Mining quorum sensing regulated proteins - Role of bacterial cell-to-cell communication in global gene regulation as assessed by proteomics. Proteomics 11: 3070-3085

Ferreira AS, Leitao JH, Silva IN, Pinheiro PF, Sousa SA, Ramos CG, Moreira LM (2010) Distribution of cepacian biosynthesis genes among environmental and clinical *Burkholderia* strains and role of cepacian exopolysaccharide in resistance to stress conditions. Appl. Environ. Microbiol. 76: 441-450

Figurski DH, Helinski DR (1979) Replication of origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans* Proc. Natl. Acad. Sci. U. S. A. 76: 1648-1652

Fong KP, Chung WSO, Lamont RJ, Demuth DR (2001) Intra- and interspecies regulation of gene expression by actinobacillus actinomycetemcomitans LuxS. Infect. Immun. 69: 7625-7634

Fuqua C, Parsek MR, Greenberg EP (2001) Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. 35: 439-468

Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria-the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176: 269-275

Galan JE, Wolf-Watz H (2006) Protein delivery into eukaryotic cells by type III secretion machines. Nature 444: 567-573

Galyov EE, Brett PJ, DeShazer D (2010) Molecular Insights into *Burkholderia pseudomallei* and *Burkholderia mallei* Pathogenesis. Annu. Rev. Microbiol. 64: 495-517

Goo E, Kang Y, Kim H, Hwang I (2010) Proteomic analysis of quorum sensing-dependent proteins in *Burkholderia glumae*. J. Proteome Res. 9: 3184-3199

Goto K, Ohata K (1956) New bacterial diseases of rice (brown stripe and grain rot). Ann. Phytopathol. Soc. Jpn. 21: 46-47

Gotschlich A, Huber B, Geisenberger O, Togl A, Steidle A, Riedel K, Hill P, Tummler B, Vandamme P, Middleton B, Camara M, Williams P, Hardman A, Eberl L (2001) Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the Burkholderia cepacia complex. Syst. Appl. Microbiol. 24: 1-14

Grant SGN, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia-coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. U. S. A. 87: 4645-4649

Groth D, Hollier C (2011) Rice Diseases of Louisiana. In: Extension LAR (ed), Baton Rouge

Ham JH, Melanson RA, Rush MC (2011) *Burkholderia glumae*: next major pathogen of rice? Mol. Plant Pathol. 12: 329-339

Hamoen LW, Venema G, Kuipers OP (2003) Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology-Sgm 149: 9-17

Holden MTG, Titball RW, Peacock SJ, Cerdeno-Tarraga AM, Atkins T, Crossman LC, Pitt T, Churcher C, Mungall K, Bentley SD, Sebaihia M, Thomson NR, Bason N, Beacham IR, Brooks K, Brown KA, Brown NF, Challis GL, Cherevach I, Chillingworth T, Cronin A, Crossett B, Davis P, DeShazer D, Feltwell T, Fraser A, Hance Z, Hauser H, Holroyd S, Jagels K, Keith KE, Maddison M, Moule S, Price C, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Simmonds M, Songsivilai S, Stevens K, Tumapa S, Vesaratchavest M, Whitehead S, Yeats C, Barrell BG, Oyston PCF, Parkhill J (2004) Genornic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. U. S. A. 101: 14240-14245

Hwang IY, Li PL, Zhang LH, Piper KR, Cook DM, Tate ME, Farrand SK (1994) TraI, a LuxI homolog, is responsible for production of conjugation factor, the Ti plasmid N-acyl homoserine lactone autoinducer. Proc. Natl. Acad. Sci. U. S. A. 91: 4639-4643

Iiyama K, Furuya N, Takanami Y, Matsuyama N (1995) A role of phytotoxin in virulence of *Pseudomonas glumae*. Ann. Phytopathol. Soc. Jpn. 61: 470-476

Jacobs JL, Fasi AC, Ramette A, Smith JJ, Hammerschmidt R, Sundin GW (2008) Identification and onion pathogenicity of Burkholderia cepacia complex isolates from the onion rhizosphere and onion field soil. Appl. Environ. Microbiol. 74: 3121-3129

Janisiewicz W, Yourman L, Roitman J, Mahoney N (1991) Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of *Pseudomonas cepacia*. Plant Dis. 75: 490-494

Jeong Y, Kim J, Kim S, Kang Y, Nagamatsu T, Hwang I (2003) Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. Plant Dis. 87: 890-895

Jung WS, Lee J, Kim MI, Ma J, Nagamatsu T, Goo E, Kim H, Hwang I, Han J, Rhee S (2011) Structural and functional analysis of phytotoxin toxoflavin-degrading enzyme. Plos One 6

Kang Y, Kim J, Kim S, Kim H, Lim JY, Kim M, Kwak J, Moon JS, Hwang I (2008) Proteomic analysis of the proteins regulated by HrpB from the plant pathogenic bacterium *Burkholderia glumae*. Proteomics 8: 106-121

Kim J, Kang Y, Choi O, Jeong Y, Jeong JE, Lim JY, Kim M, Moon JS, Suga H, Hwang I (2007) Regulation of polar flagellum genes is mediated by quorum sensing and FlhDC in *Burkholderia glumae*. Mol. Microbiol. 64: 165-179

Kim J, Kim JG, Kang Y, Jang JY, Jog GJ, Lim JY, Kim S, Suga H, Nagamatsu T, Hwang I (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. Mol. Microbiol. 54: 921-934

Kim J, Oh J, Choi O, Kang Y, Kim H, Goo E, Ma J, Nagamatsu T, Moon JS, Hwang I (2009) Biochemical evidence for ToxR and ToxJ binding to the *tox* operons of *Burkholderia glumae* and mutational analysis of ToxR. J. Bacteriol. 191: 4870-4878

King EB, Parke JL (1993) Biocontrol of *Aphanomyces* root-rot and *Pythium* damping-off by *Pseudomonas cepacia* AMMD on 4 pea cultivars. Plant Dis. 77: 1185-1188

Kohler T, Curty LK, Barja F, van Delden C, Pechere JC (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. 182: 5990-5996

Langlotz C, Schollmeyer M, Coplin DL, Nimtz M, Geider K (2011) Biosynthesis of the repeating units of the exopolysaccharides amylovoran from *Erwinia amylovora* and stewartan from *Pantoea stewartii*. Physiol. Mol. Plant Pathol. 75: 163-169

Lariceman (2010) Field Notes, Newpath injury, panicle blight. pp August 5, 2010. http://lariceman.wordpress.com/2010/2009/2009/august-2015-2010-field-notes-newpath-injury-panicle-blight/

Latuasan HE, Berends W (1961) On the origin of the toxicity of toxoflavin. Biochim. Biophys. Acta. 52: 502-508

Leo E (2006) Quorum sensing in the genus Burkholderia. Int. J. Med. Microbiol. 296: 103-110

Lewenza S, Conway B, Greenberg EP, Sokol PA (1999) Quorum sensing in *Burkholderia cepacia*: Identification of the LuxRI homologs CepRI. J. Bacteriol. 181: 748-756

Lewenza S, Sokol PA (2001) Regulation of ornibactin biosynthesis and N-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. J. Bacteriol. 183: 2212-2218

Liu H, Coulthurst SJ, Pritchard L, Hedley PE, Ravensdale M, Humphris S, Burr T, Takle G, Brurberg MB, Birch PRJ, Salmond GPC, Toth IK (2008) Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. PLoS Pathog. 4

Lutter E, Lewenza S, Dennis JJ, Visser MB, Sokol PA (2001) Distribution of quorum-sensing genes in the Burkholderia cepacia complex. Infect. Immun. 69: 4661-4666

Luvizotto DM, Marcon J, Andreote FD, Dini-Andreote F, Neves AAC, Araujo WL, Pizzirani-Kleiner AA (2010) Genetic diversity and plant-growth related features of *Burkholderia* spp. from sugarcane roots. World J. Microbiol. Biotechnol. 26: 1829-1836

Mae A, Montesano M, Koiv V, Palva ET (2001) Transgenic plants producing the bacterial pheromone N-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. Mol. Plant-Microbe Interact. 14: 1035-1042

Mahenthiralingam E, Urban TA, Goldberg JB (2005) The multifarious, multireplicon Burkholderia cepacia complex. Nat. Rev. Microbiol. 3: 144-156

Malott RJ, O'Grady EP, Toller J, Inhulsen S, Eberl L, Sokol PA (2009) A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. J. Bacteriol. 191: 2447-2460

Matsuda I, Sato Z (1988) Regulation between pathogenicity and pigment productivity in the causal agent of bacterial grain rot of rice. Ann. Phytopathol. Soc. Jpn. 54: 378

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart G, Williams P (1997) Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology-Uk 143: 3703-3711

More MI, Finger LD, Stryker JL, Fuqua C, Eberhard A, Winans SC (1996) Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science 272: 1655-1658

Nagamatsu T (2002) Syntheses, transformation, and biological Activities of 7-azapteridine antibiotics: Toxoflavin, fervenulin, reumycin and their analogues. ChemInform 33: 261-261

Nandakumar R, Shahjahan AKM, Yuan XL, Dickstein ER, Groth DE, Clark CA, Cartwright RD, Rush MC (2009) *Burkholderia glumae* and *B. gladioli* cause bacterial panicle blight in rice in the southern United States. Plant Dis. 93: 896-905

Ng WL, Bassler BL (2009) Bacterial quorum-sensing network architectures. Annu. Rev. Genet. 43: 197-222

Oh HS, Kvitko BH, Morello JE, Collmer A (2007) Pseudomonas syringae lytic transglycosylases coregulated with the type III secretion system contribute to the translocation of effector proteins into plant cells. J. Bacteriol. 189: 8277-8289

Parke JL, Gurian-Sherman D (2001) Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annu. Rev. Phytopathol. 39: 225-258

Quinones B, Dulla G, Lindow SE (2005) Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 18: 682-693

Rajinikanth J, Balaji V, Gaikwad P, Muthusami JC (2008) Melioidosis of the parotid: The tip of the iceberg. Otolaryngol. Head Neck Surg. 139: 731-732

Ried JL, Collmer A (1987) An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-eviction mutagenesis. Gene 57: 239-246

Saddler GS (1994) IMI descriptions of fungi and bacteria. 1219. *Burkholderia glumae*. Mycopathologia 128: 59-60

Saile E, McGarvey JA, Schell MA, Denny TP (1997) Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87: 1264-1271

Schaad NW, Jones JB, Chun W (2001) Laboratory guide for identification of plant pathogenic bacteria, third edition. The American Phytopathological Society Press, St. Paul, MN, USA

Shahjahan AKM, Rush MC, Groth D, Clark C (2000b) Panicle blight. Rice J. 15: 26-29

Shingu Y, Yoneyama K (2004) Essential regulator gene *tox*R for toxoflavin biosynthesis of *Burkholderia glumae*. J. Gen. Plant. Pathol. 70: 108-114

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Nature Biotechnol. 1: 784–791

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-764

Sokol PA, Sajjan U, Visser MB, Gingues S, Forstner J, Kooi C (2003) The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. Microbiology-Sgm 149: 3649-3658

Solomon JM, Lazazzera BA, Grossman AD (1996) Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. Genes Dev. 10: 2014-2024

Song Y, Xie C, Ong YM, Gan YH, Chua KL (2005) The BpsIR quorum-sensing system of Burkholderia pseudomallei. J. Bacteriol. 187: 785-790

Suzuki F, Sawada H, Azegami K, Tsuchiya K (2004) Molecular characterization of the tox operon involved in toxoflavin biosynthesis of *Burkholderia glumae*. J. Gen. Plant. Pathol. 70: 97-107

Tsushima S (1996) Epidemiology of bacterial grain rot of rice caused by *Pseudomonas glumae*. Jarq - Jpn. Agric. Res. Q. 30: 85-89

Vandamme PVP, Dawyndt P (2011) Classification and identification of the *Burkholderia cepacia* complex: past, present and future. Syst. Appl. Microbiol. 34: 87-95

von Bodman SB, Majerczak DR, Coplin DL (1998) A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. Proc. Natl. Acad. Sci. U. S. A. 95: 7687-7692

Watson WT, Minogue TD, Val DL, von Bodman SB, Churchill MEA (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol. Cell 9: 685-694

Weinberg JB, Alexander BD, Majure JM, Williams LW, Kim JY, Vandamme P, LiPuma JJ (2007) *Burkholderia glumae* infection in an infant with chronic granulomatous disease. J. Clin. Microbiol. 45: 662-665

Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T, Arakawa M (1992) Proposal of *Burkholderia* gen. nov. and transter of 7 species of the genus *Pseudomonas* homology group-II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36: 1251-1275

Yuan X (2004) Identification of bacterial pathogens causing panicle blight of rice in louisiana. Plant Pathology and Crop Physiology. Louisiana State University, Baton Rouge

Zeigler RS, Alvarez E (1989) Discoloration of rice caused by *Pseudomonas glumae* in Latin America. Plant Dis. 73: 368

Appendix. The PCR Programs and Primers Used in the Mutagenesis Studies

Amplified region	Product length	Primers (forward & reverse)	Program steps
Internal fragment of *tofI* gene	462 *bp*	tofI-vv1: GGCAGCTGCCGTCCGAAGAC tofI-vv2: CCAGCGTCTGCGCGTCGATG (Devescovi et al. 2007)	Initialization step: 95 ℃ for 2 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 60 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 40 seconds Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
Upstream flanking region of *tofI*	545 *bp*	dtofI1: ACTGGTACCTCGAACCCGACTCCG dtofI2: GGATCCAGCTCGGCGGCGATATGG	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 60 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
Downstream flanking region of *tofI*	512 *bp*	dtofI3: GGATCCACATCGACGCGCAGACGC dtofI4: GCACTAGTATCCGCCCGAGATCCG	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 62 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
Downstream flanking region of *tofR*	892 *bp*	TRD3: GGATCCGCGCGAACGCGAGGTGC TRD6: ACTAGTACGGCGTGACCGGCGTC	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 65 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
Upstream flanking region of *tofR*	426 *bp*	TofR BF: AGGATCCGCTGCTCGTTTTCC TofR BR: GACTAGTATCAGATTGCTGCG	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 55 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃

Appendix continued

tofI/*tofR*/*orf1* and their adjacent region	2456*bp* in wild type; 586 *bp* in LSUPB139	TofI(H)F: GTTCGTCAACGACG ACTACG TofR(H)R: CATGAGCATGGAAA GAGCA	Initialization step: 95 ℃ for 2 minutes Denaturation step: 94 ℃ for 1 minute Annealing step: 53 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 2.5 minutes (1 minute used for mutants) Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
tofI and its adjacent region	800 *bp* in wild type; 318 *bp* in LSUPB145; No band in LSUPB214 and LSUPB220	TofI(H)F: GTTCGTCAACGACG ACTACG TofI(H)R: CGGAATTACCACGA GGACAC	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 54 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃
tofR and its adjacent region	911 *bp* in wild type; 436 *bp* in LSUPB169	TofR(H)F: AAGAATGACAGCGT GGAAGC TofR(H)R: CATGAGCATGGAAA AGAGCA	Initialization step: 95 ℃ for 5 minutes Denaturation step: 94 ℃ for 30 seconds Annealing step: 50 ℃ for 30 seconds Extension/elongation step: 72 ℃ for 1 minute Final elongation: 72 ℃ for 7 minutes Final hold: 4 ℃

GGATCC : *Bam*HI restriction site
ACTAGT : *Spe*I restriction site

Vita

Ruoxi Chen was born at Heze city, Shandong province, China at in 1986. She went to primary school and junior high in her hometown city. She spent her high school in Shandong Heze No.1 Middle School from 2000 to 2004, and afterwards entered college in Taian city. In 2008, she attained her Bachelor of Science in Plant Protection, Shandong Agricultural University. During college time, she gained scholarship every semester and Academic Outstanding Student Prize in 2006. She was active member in young volunteers association and reading and debating club, also the secretary of university student autonomy committee. In 2008 fall, she enrolled in the plant pathology and crop physiology department at Louisiana State University as a doctoral Candidate. She presented at APS Southern Division Meeting in 2011, which was about the genomic study of quorum sensing system in *B. glumae* strain 336gr-1.