농학박사학위논문

Burkholderia glumae 균의 quorum sensing 의존 단백질체 분석과 생장 정지기 스트레스에 대한 예측

Quorum sensing-dependent proteomes and anticipation of stationary-phase stress in *Burkholderia glumae*

2013년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

구 은 혜

Quorum sensing-dependent proteomes and anticipation of stationary-phase stress in *Burkholderia glumae*

A dissertation submitted in partial

fulfillment of the requirement for

the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of

Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

Eunhye Goo

February, 2013

농학박사학위논문

Burkholderia glumae 균의 quorum sensing 의존 단백질체 분석과 생장 정지기 스트레스에 대한 예측

지도교수 황 인 규

이 논문을 농학박사학위논문으로 제출함

2013년 2월

서울대학교 대학원
농생명공학부 식물미생물학전공

구 은 혜

구 은 혜의 박사학위논문을 인준함

2013년 2월

위 원 장 이 인 원

부위원장 황 인 규

위 원 윤 제 선

위 원 이 상 기

위 원 이 용 환

A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Quorum sensing-dependent proteomes and anticipation of stationary-phase stress in *Burkholderia glumae*

UNDER THE DIRECTION OF DR. INGYU HWANG

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

BY
EUNHYE GOO

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

FEBRUARY 2013

APPROVED AS A QUALIFIED THESIS OF EUNHYE GOO
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
BY THE COMMITTEE MEMBERS

CHAIRMAN Lim-Won Lee

VICE CHAIRMAN Hwang Ingyu

MEMBER Joe Sun Moon

MEMBER Sangkee Rhee

MEMBER Yong-Hwan Lee

Quorum sensing-dependent proteomes and anticipation of stationary-phase stress in *Burkholderia glumae*

Eunhye Goo

ABSTRACT

Burkholderia glumae, the causative agent for bacterial rice grain rot, has a LuxR-LuxI type quorum sensing (QS) system. The bacterium utilizes *N*-octanoyl homoserine lactone synthesized by TofI and its cognate receptor TofR to activate expression of genes for toxoflavin biosynthesis and an IclR-type transcriptional regulator gene, *qsmR*. Since QS is essential for pathogenicity of *B. glumae*, we analyzed QS-dependent proteome by two-dimensional gel electrophoresis and ESI-MS/MS. We found that a total of 79 proteins, including previously known QS-depedent proteins, were differentially expressed between the wild-type BGR1 and the *tofI* mutant BGS2 strains. Among this set, 59 proteins were found in the extracellular fraction, and 20 were cytoplasmic. The extracellular 34 proteins including protease, lipase, phosphatases, were secreted through the type II secretion system (T2SS). Real-time RT-PCR analysis showed that the corresponding genes of the 49

extracellular and 13 intracellular proteins are regulated by QS at the transcriptional level. The T2SS, encoded by 12 general secretion pathway (*gsp*) genes with three independent transcriptional units, was controlled by QS. β-Glucuronidase activity analysis of *gsp*::Tn*3*-*gusA* gene fusions and electrophoretic mobility shift assays revealed that the QsmR directly regulates the expression of *gsp* genes. The T2SS defective mutants were less virulent than the wild type in rice panicles, indicating that the T2SS-dependent extracellular proteins play important roles in *B. glumae* virulence.

Acyl-homoserine lactone-mediated QS regulates diverse activities in many species of *Proteobacteria*. QS-controlled genes commonly code for production of secreted or excreted public goods. QS affords a means of population density-dependent gene regulation. Control of public goods via QS provides a fitness benefit. Another potential role for QS is to anticipate overcrowding. As population density increases and stationary phase approaches, QS might induce functions important for existence in stationary phase. Here we provide evidence that in two related species of the genus *Burkholderia* QS allows individuals to anticipate and survive stationary phase stress, base toxicity. Survival requires QS-dependent activation of cellular enzymes required for production of excreted oxalate, which serves to counteract ammonia-mediated alkaline toxicity during stationary phase. Our findings

provide an example where QS can serve as a means to anticipate stationary phase or life at the carrying capacity of the population by activating expression of cytoplasmic enzymes, altering cellular metabolism and producing a shared resource or public good, oxalate.

KEY WORDS: *Burkholderia glumae*, Quorum sensing, Proteomics, T2SS, Stationary phase stress, Oxalate

Student number: 2006-23279

CONTENTS

LIST OF TABLES

page

CHAPTER 1

CHAPTER 2

LIST OF FIGURES

page

CHAPTER 1

CHAPTER 2

INTRODUCTION

Many bacterial phenotypes are controlled in the cell density-dependent manner of quorum sensing (QS) (Miller *et al*., 2001; Whitehead *et al*., 2001). *N*-acyl homoserine lactones (HSLs) are widespread QS signals among Gram-negative bacteria, and bind to their cognate receptor LuxR-type transcription factors (Miller *et al*., 2001; Whitehead *et al*., 2001). Phenotypes controlled by QS include virulence factor production, symbiosis, biofilm formation, motility, conjugation, competence, antibiotic production, and sporulation (Miller *et al*., 2001; Hammer *et al*., 2003; Danhorn *et al*., 2007). In addition, QS systems are often important for successful bacterial colonization and pathogenesis within the bacteria's hosts (Parker *et al*., 2009; Ulrich *et al*., 2004; Passador *et al*., 1993; Winson *et al*., 1995; von Bodman *et al*., 2008). Thus, QS-defective mutants are substantially less virulent than the wild-type strains in many pathogenic bacteria (Parker *et al*., 2009; Ulrich *et al*., 2004; Passador *et al*., 1993; Winson *et al*., 1995; von Bodman *et al*., 2008).

Among the multifunctional roles of QS in Gram-negative bacteria, QS control of protein secretion is relatively uncharacterized. The most broadly conserved protein secretion system in Gram-negative bacteria is the T2SS, which is responsible for exporting signal sequence-dependent exoproteins

across the two membranes (Robert *et al*., 2005; Sandkvist *et al*., 2001). The importance of T2SS is well demonstrated in *Pseudomonas aeruginosa* in which important virulence factors (e.g., exotoxin A, elastase, and lipases) are secreted via a T2SS (Robert *et al*., 2005; Sandkvist *et al*., 2001). The T2SS of *P. aeruginosa* is encoded by 12 *xcp* genes whose expression is regulated by QS (Chapon-Herve *et al*., 1997). Therefore, the percentage of QS-dependent secreted proteins is significantly higher than that of cellular or surface proteins (Arevalo-Ferro *et al*., 2003). The protein secretion systems and regulation of genes involved in protein secretion in *Burkholderia* species are poorly understood.

In *P. aeruginosa* PAO1, QS-regulated genes and proteins have been heavily studied at both the transcriptional and post-transcriptional levels based on genomic information (Arevalo-Ferro *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003). Even though transcriptome analysis is sensitive and global tool to catalog genome-wide information of QS-dependent genes, it does not report the final state of the QS-regulated proteins because some proteins undergo post-translational modifications (Gygi *et al*., 1999).

Therefore, proteomic analysis has become an additional choice to search for proteins under the control of QS at post-transcriptional levels. For example, novel QS-regulated proteins were identified by proteomic analysis, in

addition to the QS-controlled genes previously identified by transcriptome analysis, in *P. aeruginosa* (Arevalo-Ferro *et al*., 2003). Another genome-wide screen of QS-dependent genes was carried out in *B. cenocepacia* by high-throughput screening a random promoter library (Subsin *et al*., 2007). Although these global analyses have generated substantial amounts of novel information on QS, the QS-dependent gene data are still very limited in *Burkholderia* species because only a few bacterial strains of *Burkholderia* have been studied.

We study QS-mediated gene regulation of *B. glumae*, the organism responsible for bacterial rice grain rot and wilt in many field crops. This bacterium possesses QS systems that utilize *N*-octanoyl homoserine lactone (C8-HSL), synthesized by TofI, as a QS signal molecule (Kim *et al*., 2004). TofR is the cognate C8-HSL receptor, and its complex with C8-HSL activates the expression of toxoflavin biosynthesis and transport genes and an IclR-type transcriptional regulator gene, *qsmR* (Kim *et al*., 2004; Kim *et al*., 2007). Flagella biosynthetic genes and a major catalase gene (*katG*) also belong to the QsmR regulon (Kim *et al*., 2007; Chun *et al*., 2009).

In this study, we carried out a proteomic analysis of QS-dependent proteins in *B. glumae* and compared extracellular and cellular proteins in the wild type BGR1 and the *tofI* mutant BGS2 strains using 2-DE and ESI-MS/MS.

The fact that the *tofI* mutant BGS2 strain exhibited significantly reduced amounts of extracellular proteins compared to the wild type was due to direct regulation of general secretion pathway genes (*gsp*) for the T2SS by QsmR. We also report that *gsp* genes in *B. glumae* are organized as three independent transcriptional units. Mutations in each *gsp* gene conferred reduced disease severity, indicating that the T2SS-dependent extracellular proteins are important for full virulence.

QS-controlled genes commonly code for production of extracellular public goods that can be shared by all members of the group regardless of which members produce them. For examples, the QS-dependent extracellular signals in *V. fischeri* were used to control nutrient acquisition strategies in different environment (Studer *et al*., 2008). In mixed microbial habitat, QS control of antibiotic production is important for interspecies competition for limited resources (Chandler *et al*., 2012). The extracellular products from *Pseudomonas aeruginosa* have significant effects on virulence, and 3OC12-HSL produced by *P. aeruginosa* activates multiple cell types and thus potentially change the host response during infections (Smith *et al*., 2003). In *Pseudomonas aeruginosa*, fitness benefits provided by QS are relatively greater at higher cell densities because the QS-dependent extracellular public goods, proteases are produced only when they can be used efficiently (Darch *et al*.,

2012). Additional potential roles of QS in bacteria have been proposed and include the hypothesis that QS enables bacteria to anticipate population carrying capacity in a given environment. Anticipation of stationary phase might allow individuals to modify their physiology in preparation for survival at population carrying capacity.

Here we address the question of whether QS is involved in anticipation of stationary-phase stress in two closely related bacteria: the rice pathogen *Burkholderia glumae*, and the non-pathogenic saprophyte *B. thailandensis*. Each species contains a conserved *N*-octanoyl homoserine lactone (C8-HSL) signaling system. In *B. glumae*, TofI-R regulates toxoflavin production, motility, protein secretion, and QsmR, an IclR-type transcriptional regulator (Kim *et al*., 2004; Kim *et al*., 2007; Goo *et al*., 2010). Less is known about gene control by C8-HSL in *B. thailandensis* via BtaI-R (Chandler *et al*., 2009). In the present study, we found QS induces cellular enzymes for production of oxalate (HOOC-COOH), which is excreted into the culture medium and is likely shared by other members of the population and as such is a public good. The oxalate protects stationary-phase cells from self-intoxication and killing as a result of ammonia production.

LITERATURE CITED

Arevalo-Ferro C, Hentzer M, Reil G, Gorg A, Kjelleberg S, Givskov M, Riedel K, Eberl L (2003) Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol* 5:1350-1369.

Chandler JR, et al. (2009) Mutational analysis of *Burkholderia thailandensis* quorum sensing and self-aggregation. *J Bacteriol* 191:5901-5909.

Chandler JR, Heilmann S, Mittler JE, Greenberg EP (2012) Acyl-homoserine lactone-dependent eavesdropping promotes competition in a laboratory co-culture model. *ISME J* doi: 10.1038/ismej.2012.69.

Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. *Annu Rev Microbiol* 61:401-422.

Darch SE, West SA, Winzer K, Diggle SP (2012) Density-dependent fitness benefits in quorum sensing bacterial populations. *Proc Natl Acad Sci USA* 109:8259-8263.

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.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720-1730.

Hammer BK, Bassler BL (2003) Quorum sensing controls biofilm formation in *Vibrio cholera*. *Mol Microbiol* 50:101-104.

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.

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.

Parker CT, Sperandio V (2009) Cell-to-cell signalling during pathogenesis. *Cell Microbiol* 11:363-369.

Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260:1127-1130.

Robert V, Filloux A, Michel GP (2005) Role of XcpP in the functionality of the *Pseudomonas aeruginosa* secreton. *Res Microbiol* 156:880-886.

Sandkvist M (2001) Type II secretion and pathogenesis. *Infect Immun* 69:3523-3535.

Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185:2066-2079.

Smith RS, Iglewski BH (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* 6:56-60.

Studer SV, Mandel MJ, Ruby EG (2008) AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. *J Bacteriol* 190:5915-5923.

Subsin B, Chambers CE, Visser MB, Sokol PA (2007) Identification of genes regulated by the *cepIR* quorum-sensing system in *Burkholderia cenocepacia* by high-throughput screening of a random promoter library. *J Bacteriol* 189:968-979.

Ulrich RL, et al. (2004) Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *J Med Microbiol* 53:1053-1064.

von Bodman SB, Willey JM, Diggle SP (2008) Cell-cell communication in bacteria: united we stand. *J Bacteriol* 190:4377-4391.

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185:2080-2095.

Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC (2001) Quorum-sensing in gram-negative bacteria. *FEMS Microbiol Rev* 25:365–404.

Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW, Lazdunski A, Stewart GSAB, Williams P (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92:9427-9431.

CHAPTER 1

Proteomic Analysis of Quorum Sensing-Dependent Proteins in The Plant Pathogenic Bacterium *Burkholderia glumae*

ABSTRACT

Burkholderia glumae causes rice grain rot and has a LuxR-LuxI type quorum sensing (QS) system. The bacterium utilizes *N*-octanoyl homoserine lactone synthesized by TofI and its cognate receptor TofR to activate expression of an IclR-type transcriptional regulator gene, *qsmR* and genes for toxoflavin biosynthesis. Since QS is essential for pathogenicity of *B. glumae*, we analyzed proteomes by two-dimensional gel electrophoresis and ESI-MS/MS to identify differentially expressed proteins by QS. We found that a total of 79 proteins are differentially expressed between the wild-type BGR1 and the *tofI* mutant BGS2 strains. Among those, 59 proteins were found in the extracellular fraction, and 20 were cytoplasmic. Among the 59 extracellular proteins, 34 proteins including protease, lipase, phosphatase and elongation factor Tu were secreted through the type II secretion system (T2SS). The results of real-time RT-PCR showed that the corresponding genes of the 49 extracellular and 13 intracellular proteins are regulated by QS at the transcriptional level. To determine if the T2SS is controlled by QS in *B. glumae*, we identified the twelve general secretion pathway (*gsp*) genes responsible for the T2SS and analyzed their gene expression. The *gsp* genes are organized as three independent transcriptional units as determined by RT-PCR. β-Glucuronidase activity analysis of

gsp::Tn*3*-*gusA* gene fusions revealed that the expression of *gsp* genes are regulated by QS. Electrophoretic mobility shift assays showed that the QsmR binds to the promoter regions of the three independent transcriptional units, which confirmed that QsmR directly regulates the expression of *gsp* genes. The T2SS defective mutants were less virulent than the wild type in rice panicles, indicating that the T2SS-dependent extracellular proteins play important roles in *B. glumae* virulence.

Contents of this chapter have been published in Journal of Proteome Research (Goo *et al*. 2010. Proteomic analysis of quorum sensing-dependent proteins in *Burkholderia glumae*. *J Proteome Res* 9: 3184-3199).

INTRODUCTION

Quorum sensing (QS) is the regulation of gene expression in response to the bacterial cell population density (Miller *et al*., 2001; Whitehead *et al*., 2001). The cell density reflects the accumulation of the signal molecule that is produced from QS bacteria to a critical threshold concentration. In Gram-negative bacteria, QS is often mediated by *N*-acyl homoserine lactones (HSLs), which are synthesized by the LuxI-type signal synthase. The signal molecules differ with respect to the length, saturation and substitutions of the side-chains and bind to their cognate receptor LuxR-type transcription factors resulting in altered gene expression (Miller *et al*., 2001; Whitehead *et al*., 2001). Phenotypes controlled by QS include virulence factor production, symbiosis, biofilm formation, motility, conjugation, competence, antibiotic production, and sporulation (Miller *et al*., 2001; Whitehead *et al*., 2001; Danhorn *et al*., 2007). In addition, QS systems are often important for successful bacterial colonization and pathogenesis within the bacteria's hosts (Parker *et al*., 2009; Ulrich *et al*., 2004; Passador *et al*., 1993; Winson *et al*., 1995; von Bodman *et al*., 2008). Thus, QS-defective mutants are substantially less virulent than wild-type strains in many pathogenic bacteria (Parker *et al*., 2009; Ulrich *et al*., 2004; Passador *et al*., 1993; Winson *et al*., 1995; von Bodman *et al*., 2008).

While QS is known to involve in diverse activities in Gram-negative bacteria, QS control of protein secretion is relatively uncharacterized. Protein secretion systems controlled by QS include Type I, II (T2SS), III, and VI (T6SS) systems in plant pathogen *Pectobacterium atrosepticum* (Liu *et al*., 2008). The T2SS that is responsible for exporting of signal-sequence dependent exoproteins is the most broadly conserved protein secretion system in Gram-negative bacteria (Robert *et al*., 2005; Sandkvist *et al*., 2001). The importance of T2SS is well demonstrated in *Pseudomonas aeruginosa* in which important virulence factors (e.g., exotoxin A, elastase, and lipases) are secreted via a T2SS (Robert *et al*., 2005; Sandkvist *et al*., 2001). In *P. aeruginosa*, T2SS is encoded by 12 *xcp* genes whose expression is the growth-phase dependent (Chapon-Herve *et al*., 1997). Therefore, the percentage of QS-dependent secreted proteins is significantly higher than that of intracellular or surface proteins (Arevalo-Ferro *et al*., 2003). Furthermore, it was recently shown that the expression of genes for the T6SS and its predicted substrates [e.g., hemolysin-coregulated proteins (Hcp)] is regulated by QS in *P. atrosepticum* and *Aeromonas hydrophila* (Liu *et al*., 2008; Khajanchi *et al*., 2009). The protein secretion systems and regulation of genes involved in protein secretion in *Burkholderia* species are poorly understood.

Analysis of transcriptomics and post-transcriptional modifications

proteomics based on the genome sequencing data systematically map the quorum-sensing regulon in *P. aeruginosa* PAO1 (Arevalo-Ferro *et al*., 2003; Schuster *et al*., 2003; Wagner *et al*., 2003). According to two representative QS-dependent transcriptome analyses of *P*. *aeruginosa* PAO1, expression of QS-controlled genes is dependent upon growth phase and environment (Schuster *et al*., 2003; Wagner *et al*., 2003). Most such genes are induced or repressed at the transition period between the exponential and stationary phases (Schuster *et al*., 2003). Even though transcriptome analysis is feasible to catalog global gene expression patterns modulate by QS regulons, it has some limitations due to no conclusion on the post-translational modification state (Gygi *et al*., 1999).

Therefore, proteomic analysis has become an additional choice to search for proteins under the control of QS at post-transcriptional levels. For example, novel QS-regulated proteins were identified by proteomic analysis, in addition to the QS-controlled genes previously identified by transcriptome analysis, in *P. aeruginosa* (Arevalo-Ferro *et al*., 2003). However, this method also cannot determine a whole proteomes completely; detection sensitivity is relatively low, and insoluble proteins are easily lost during preparation (Arevalo-Ferro *et al*., 2003). In another genome-wide screening, QS-dependent genes were identified by high-throughput screening a random promoter library of *B. cenocepacia*

(Subsin *et al*., 2007). Although these global analyses have generated substantial amounts of novel information on QS, the QS-dependent gene data are still very limited in *Burkholderia* species because only a few bacterial strains of *Burkholderia* have been studied.

We use a model organism, *B. glumae* that is responsible for bacterial rice grain rot and wilt in many field crops to study QS-mediated gene regulation. This bacterium possesses LuxR-LuxI type QS systems that utilize *N*-octanoyl homoserine lactone (C8-HSL), synthesized by TofI, as a QS signal molecule (Kim *et al*., 2004). TofR is the cognate C8-HSL receptor, and its complex with C8-HSL activates the expression of toxoflavin biosynthesis and transport genes and an IclR-type transcriptional regulator gene, *qsmR* (Kim *et al*., 2004; Kim *et al*., 2007). Flagella biosynthetic genes and a major catalase gene (*katG*) also belong to the QsmR regulon (Kim *et al*., 2007; Chun *et al*., 2009).

Recently the complete genome sequence of *B. glumae* BGR1 was reported, and 5,776 predicted open reading frames (ORF) were annotated (Lim *et al*., 2009). However, complete lists of the TofR and QsmR regulons in *B. glumae* have not yet been reported. In this study, we carried out a proteomic analysis of QS-dependent proteins in *B. glumae* and compared extracellular and cellular proteins in the wild type BGR1 and the *tofI* mutant BGS2 strains using 2-DE and ESI-MS/MS. The fact that the *tofI* mutant BGS2 strain exhibited

significantly reduced amounts of extracellular proteins compared to the wild type was due to direct regulation of general secretion pathway genes (*gsp*) for the T2SS by QsmR. We also report that *gsp* genes in *B. glumae* are organized as three independent transcriptional units. Mutations in each *gsp* gene conferred reduced disease severity, indicating that the T2SS-dependent extracellular proteins are important for full virulence.

MATERIALS AND METHODS

I. Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. All of the *B. glumae* strains in this study were grown to exponential phase in Luria–Bertani (LB) medium (1% w/v tryptone and 0.5% w/v yeast extract, pH 7.0; USB Corp., Cleveland, OH, USA) at 37˚C with shaking for 12 h. The concentration of C8-HSL produced by the wild-type strain was approximately 60 nM at the time of sampling as previously reported (Kim *et al*., 2007). Antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 20 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; nalidixic acid, 20 μg mL^{-1}; rifampicin, 50 μg mL^{-1}; spectinomycin, 25 μg mL^{-1}; and tetracycline, 10 μg mL^{-1}.

II. Nucleic acid manipulations.

Standard methods were used for DNA cloning, restriction mapping, and gel electrophoresis (Sambrook *et al*., 2001). Vector DNA was treated with the appropriate restriction enzymes as recommended by the supplier (New England Biolabs, Ipswich, MA, USA), and extraction of the DNA fragments from the gels was carried out as described by the manufacturer (Qiagen, Valencia, CA,

USA). Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The residual DNA in the isolated RNA samples was removed with a DNA-Free kit (Ambion, Austin, TX, USA). The pLAFR3 and pLAFR6 derivatives were mobilized into the *B. glumae* strains by triparental mating (Figurski *et al*., 1979).

III. Transposon mutagenesis, marker-exchange, and Southern hybridization.

The cosmid clone COS_G_BGR1_02_H06 (KROPBASE: http://kropbase.snu.ac.kr/cgi-bin/bglumae/blast_bg.cgi) carrying all of the *B. glumae* BGR1 *gsp* genes was renamed pPW2 and mutagenized with Tn*3-gusA*, as described previously (Bonas *et al*., 1989). The orientation and insertion site of Tn*3-gusA* in each mutant were determined by restriction enzyme digestions and direct sequencing of the plasmid using the primer Tn3-gus (5'-CCGGTCATCTGAGACCATTAAAAGA-3'). The mutagenized plasmids carrying Tn*3-gusA* insertions were individually introduced into the wild-type BGR1, the *tofI* mutant BGS2, and the *qsmR* mutant BGS9 strains by conjugation, followed by marker-exchange into the chromosome, as described previously (Ruvkun *et al*., 1981). All marker-exchanges were confirmed by Southern hybridization analysis as described previously (Sambrook *et al*.,

2001).

IV. Protein sample preparation for 2-DE.

For cellular protein preparation, bacterial cell pellets were washed twice and resuspended with 20 mM Tris-HCl, pH 7.4 and lysed by sonication with a VCX-750 sonicator (Sonics & Materials Inc., Newton, CT, USA). The cellular proteins were precipitated with 10% w/v trichloracetic acid (TCA) followed by centrifugation at 12,000 × *g* for 30 min at 4˚C. To harvest proteins from the culture supernatants, the cells were removed by centrifugation at 10,000 × *g* for 20 min at 4˚C, followed by filtering through a 0.45-μm pore-size membrane filter. Extracellular proteins were precipitated with 10% w/v TCA, as described above. The pellets were washed several times with cold ethanol, dried, and stored at -80˚C. The protein concentration of each sample was determined by Bradford assay using BSA as a standard (Bradford *et al*., 1976).

V. 2-DE.

A total of 600 μg (for Coomassie blue staining) or 80 μg (for silver staining) of each protein sample was resuspended in 450 μl of rehydration

solution [2% w/v CHAPS, 2% v/v immobilized pH gradient (IPG) buffer (GE Healthcare, Buckinghamshire, England), 100 mM DTT, 8 M urea, and 0.002% w/v bromophenol blue]. The proteins were focused in the first dimension using 24 cm, pH 3-10 or pH 4–7 IPG strips (GE Healthcare). Isoelectric focusing was then performed using an Ettan IPGphor (GE Healthcare) at a constant temperature of 20˚C with a total of 74,500 Vh as follows: 80 V for 1 h; 500 V for 1 h; 1,000 V for 1 h; and 8,000 V up to 74,500 Vh. The strips were equilibrated before running the second gel, as described previously (Gorg *et al*., 1995). SDS-PAGE was performed using an Ettan DALTsix Large Vertical Electrophoresis System (GE Healthcare). Samples were separated in 12.5% *T* acrylamide:bis-acrylamide (37.5:1) gels in the 10-100 kDa range. The proteins were stained with Coomassie Brilliant Blue G-250 or a silver staining kit (GE Healthcare), as described by the manufacturer. The reproducibility of the annotated spots was tested at least three times in repeated 2-DE experiments.

VI. 2-DE analysis and MS/MS.

Image analysis, including alignments and matching between spots, was performed using PDQuest™ 2-D Analysis Software V 8.0 (Bio-Rad, Hercules, CA, USA). The sum of the spot densities on each gel was equalized, and the average value of the adjusted spot intensities was used to compare protein spots.

The analysis of protein spots was independently repeated at least three times. The protein spots of interest were carefully excised and destained with 100 µL of destaining solution (30 mM potassium ferricyanide and 100 mM sodium thiosulfate). ESI-MS/MS of the peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The data were processed using a MassLynx ver. 3.5 Windows NT system (Micromass), and *de novo* sequencing of the fragmentation spectra of the peptides was carried out using PepSeq (Micromass). The parameters used to assign positive protein identifications are as follows: up to one missed cleavage, fixed modifications of carbamidomethyl, peptide tolerance ±0.6 Da, MS/MS tolerance ±0.6 Da, and peptide charge of 2+ or 3+. The resulting sequences were searched against the translated open reading frames (ORFs) in the *B. glumae* BGR1 genomic database in the KROPBASE. The results were only accepted when they satisfied that the *de novo* peptide sequencing data comprised at least eight matched amino acids, and the matched results were below the E-value inclusion threshold (E = 0.05). A homology search of each putative ORF with proteins present in the NCBI database was performed using the BLAST program (Altschul *et al*., 1990).

VII. Reverse transcription polymerase chain reaction (RT-PCR) and

Real-time RT-PCR analysis.

A total of 1 μg of RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) for 1 h at 42˚C. PCR reactions were performed using rTaq polymerase (Takara, Shiga, Japan) on a PTC-200 Thermo Cycler (MJ Research, Walthan, MA, USA) with the following conditions: 96˚C for 2 min, followed by 35 cycles of 96˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. The primer pairs used for RT-PCR and real-time RT-PCR are listed in Table 2, and RT-PCR products from all samples were analyzed on agarose gels. The 16S rRNA was used as a positive control. Transcriptional levels were determined by Power SYBR® Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The thermal cycling parameters were: 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 s, and 60˚C for 1 min. PCRs were repeated three times, and all data were normalized based on the 16S rRNA gene, as a reference, using a 7500 Software ver. 2.0.3 (Applied Biosystems). The mean of the fold change was calculated by dividing the mean CT of BGR1 by that of BGS2.

VIII. β-Glucuronidase assay.

β-Glucuronidase enzyme assays were performed as described

previously (Jefferson *et al*., 1987), with some modifications. Briefly, all *B. glumae* derivatives were grown in LB medium at 37˚C for 12 h with shaking. If necessary, C8-HSL was added at 1 μM when the cells were subcultured. The bacterial cells were pelleted by centrifugation, resuspended in GUS extraction buffer, and lysed using a VC750 sonicator. Extracts were then incubated with the substrate 4-methylumbelliferyl glucuronide, and fluorescence was measured at 365-395 nm for excitation and 440-470 nm for emission in a DQ300 fluorometer (Hoefer Scientific Instruments, Holliston, MA, USA). One unit of activity of β-glucuronidase was defined as 1 nm of 4-methylumbelliferone released per bacterium per minute.

IX. Over-expression and purification of QsmR.

Over-expression and purification of QsmR were performed as described previously (Kim *et al*., 2007).

X. Electrophoretic mobility shift assay (EMSA).

The 313 bp upstream of *gspD* and the 152 bp intergenic region between *gspC* and *gspG* were PCR-amplified using pPW2 as the template and the gspDp-F/gspDp-R and gspCGp-F/gspCGp-R primers, respectively. The PCR products were labeled with biotin using a Lightshift Chemiluminescent

Electrophoretic Mobility Shift Assay Kit, as described by the manufacturer (Pierce, Rockford, IL, USA). For non-specific competitor DNA, the 242 bp upstream region of *katE* was PCR-amplified using the KEN1 and KEN2 primers. Purified QsmR-His (100 nM) was incubated with 0.75 nM of biotin-labeled DNA in binding buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1 mM EDTA] for 15 min at 28˚C. For the competitor DNA, a 10-fold molar excess of unlabelled target DNA was added to the reaction along with extract prior to addition of the labeled DNA target. Aside from the specific competitor DNA, a non-specific unlabeled competitor DNA was also added to the binding reaction. The mixtures were separated on a non-denaturing 4% polyacrylamide gel and transferred to nitrocellulose membranes followed by detection with streptavidin HRP/chemiluminescence, as described by the manufacturer (Pierce).

XI. Plant inoculation.

Rice plants (*Oryza sativa* cv. Milyang 23) were grown in a greenhouse, inoculated at the flowering stage with *B. glumae* at approximately 1×10^8 colony forming units ml^{-1} using an atomizer (Binks Wren Airbrush; Binks, Glendale Heights, IL, USA), and kept in a greenhouse. The disease in the rice plants was

evaluated 7 days after inoculation, and the disease index was determined as described by Iiyama et al. (Iiyama *et al*., 1995), using the following scale: 0 = healthy panicle, 1 = panicle 0-20% discolored, 2 = panicle 20-40% discolored, 3 = panicle 40-60% discolored, 4 = panicle 60-80% discolored, and 5 = panicle 80-100% discolored. Disease index = $\sum$ (number of samples per score $\times$ score)/the total number of panicles. Pathogenicity assays were repeated three times with three replications.

RESULTS

I. Identification of QS-dependent proteins.

To identify QS-dependent proteins in *B. glumae*, we compared the protein expression pattern of the wild type strain with those of the *tofI* mutant strain grown to exponential phase in LB medium by two-dimensional gel electrophoresis (2-DE) and ESI-MS/MS. We first tried pH 3-10 IPG strips to identify differentially expressed QS-dependent proteins and found that most were present in the pH 4-7 range. Therefore, we focused on the proteins present in the pH 4–7 range in the remaining experiments.

The fold changes were calculated by comparing spot intensities of the wild type to *tofI* mutant using quantitative image analysis software. We identified a total of 79 proteins that were differentially expressed between the two strains (Table 3, 4, 5 and 6). Among those, 59 proteins were found in the extracellular fraction, and 20 were cytoplasmic proteins. A total 79 proteins were classified into four groups: i) the intensities of 46 extracellular protein spots were more than 2.0-fold reduced in the *tofI* mutant (Group I, Fig. 1 and Table 3); ii) 13 extracellular protein spots with increased intensities in the *tofI* mutant (Group II, Fig. 1 and Table 4); iii) 18 cellular proteins were upregulated

in the wild type (Group III, Fig. 1 and Table 5); and iv) two cellular proteins were downregulated by the QS system (Group IV, Fig. 1 and Table 6). Among QS-upregulated 64 proteins in Groups I and III, 30 only found in the wild type, and intensities of 34 protein spots were higher in the wild type than the *tofI* mutant (Figs. 2 and 3). Among QS-downregulated 15 proteins in Groups II and IV, 10 only appeared in the *tofI* mutant, and 5 were more abundant in the *tofI* mutant (Figs. 2 and 3). Protein spots from the *tofI* mutant showed differences with the wild type were recovered, when pBGA43 carrying the *tofI* gene was introduced in to the *tofI* mutant.

II. QS-dependent extracellular proteins and T2SS-dependent secretion of most identified extracellular proteins.

Among the identified QS-dependent extracellular proteins, subunits of flagellin (GR1, GR2, GR3, GR4, and GR20) and lipases (GR15 and GR22) were consistent with the data of Kim *et al*. (2007) and Devescovi *et al*. (2007) who showed that the proteins regulated by QS in *B. glumae*. Serine metalloprotease homologs (GR5, GR6, and GR21), proteins involved in antioxidation (GS5 and GS6), and putative membrane proteins (GR44 and GR45) have previously been shown to be QS regulated in other bacteria (Tables 3 and 4).

Moreover, we found other extracellular proteins whose functions are not fully characterized. These proteins were belonged to five categories. i) highly conserved molecular determinants, known as pathogen-associated molecular patterns (PAMPs), such as elongation factor Tu (GR8, GR30, GR33, GR35, GR36, and GR38), the DnaK molecular chaperone (GR32 and GS1) and trigger factor (GS2); ii) filamentous hemagglutinin (GR18); iii) putative enzymes (GR12, GR29, GR37, GR39, and GR42); iv) Hcp (GS12 and GS13); and v) proteins of unpredictable function (Tables 3 and 4).

Among the 59 identified extracellular proteins, 20 proteins had typical N-terminal signal peptides (Fig. 1 and Tables 3 and 4). To confirm that proteins possessed signal peptides are secreted through the T2SS, we compared the extracellular 2-DE protein spot intensities from the wild type with those of a T2SS mutant (BGPW2) (Fig. 4). Thirty-four out of the 59 identified QS-dependent extracellular proteins were secreted via T2SS (Fig. 1). The two proteins (GS3 and GS5) predicted to have a signal peptide were not present in the supernatant of the T2SS mutant (Figs. 2 and 4). Besides, we observed that 16 extracellular proteins predicted to have no signal peptides were secreted in a T2SS-dependent manner. These proteins include elongation factor Tu (GR8, GR30, GR33, GR35, GR36, and GR38), DnaK (GR32), Trp-1 (ToxA) (GR34), 3-hydroxyacyl-CoA dehydrogenase (GR39), thiamine-phosphate

pyrophosphorylase (GR42), and hypothetical proteins (GR16, GR23, GR24, GR26, GR27, and GR31) (Table 3).

III. QS-dependent cellular proteins.

The analysis of cellular fraction revealed that 20 proteins were differentially expressed between the wild type and *tofI* mutant (Fig. 3 and Tables 5 and 6). Among the identified 20 proteins, 11 proteins displayed at least 3.0-fold increase in intensity in the wild type, and 7 protein spots were found in the wild-type fraction but were missing in the *tofI* mutant fraction. We also observed that two hypothetical proteins were only identified in the *tofI* mutant fraction. Protein spots changed in the *tofI* mutant were rescued to the wild-type pattern after complementation with pBGA43 carrying the tofI gene (Fig. 3).

Consistent with previous study (Kim *et al*., 2004), expression of the ToxA (RC5) and ToxD (RC16), which are responsible for toxoflavin biosynthesis toxoflavin, is QS-regulated. We also identified QS-dependent cellular proteins; aldehyde dehydrogenase family (RC1) and malate/lactate dehydrogenase family members (RC5), two component transcriptional regulator (RC17), and eight hypothetical proteins (Table 5). Interestingly, the 3-oxoacid CoA-transferase A subunit AtoD (RC8 and GR37) and the 3-oxoadipate CoA-succinyl transferase beta subunit AtoA (RC9, RC13, and GR29), which were identified as QS-dependent extracellular proteins, were

found in the cellular fractions as well (Figs. 2 and 3).

IV. Organization of *gsp* genes.

Because the majority of QS-dependent extracellular proteins were secreted via type II secretion system, we hypothesized that expression of *gsp* genes responsible for T2SS would be activated by QS. Therefore, we first identified the *B. glumae gsp* genes from the complete genome information (KROPBASE). There are 12 *gsp* genes *gspD*, *E*, *F*, *C*, *G*, *H*, *I*, *J*, *K*, *L*, *M*, and *N* on chromosome 1 (Fig. 5). The gene IDs of these ORFs are bglu_1g00380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, and 270, respectively. The *gspC* gene is located between the *gspF* and *gspG* genes, and the orientation of *gspC* gene is opposite to other genes in the gene clusters (Fig. 5). In *B. glumae,* there is no the analogous gene of *gspO* found in the same gene clusters of *B. capacia* and *B. pseudomallei*. Based on a KROPBASE search providing the end sequence information of cosmid clones of the wild-type BGR1, pPW2 was found to carry all 12 *gsp* genes (Fig. 5).

To determine whether the *gsp* genes are polycistronic, RT-PCR was performed using nine sets of primers to amplify *gspD-E*, *E-F*, *G-H*, *H-I*, *I-J*, *J-K*, *K-L*, *L-M*, and *M-N* (Fig. 5). RT-PCR followed by Southern hybridization

indicated that *gspD* to *F*, *gspC*, and *gspG* to *N* are transcribed as independent single transcripts (Fig. 5).

V. QsmR controls *gsp* gene expression.

To confirm the hypothesis that QS regulates the expression of *gsp* genes, we measured the β-glucuronidase activities of Tn*3-gusA* fusions in the *gspD, E, F, C, G, H, J, K, L, M,* or *N* genes in the wild type, the *tofI* mutant, and the *qsmR* mutant strains. The expression levels of *gsp* genes were reduced in *tofI* mutant and the *qsmR* mutant, but it recovered to the wild-type levels after addition of 1 μM C8-HSL exogenously to *tofI* mutant culture (Table 7). These results indicated that expression of *gsp* genes is controlled by QS.

Since *β*-glucuronidase activity of *gspDEFCGHIJKLMN-gusA* transcriptional fusion in the wild type was higher than in either mutant, we confirmed that QsmR directly activated the expression of the *gsp* genes through an electrophoretic mobility shift assay (EMSA). The result showed that QsmR-His bound to the 313 bp *gspD* promoter region and the 152 bp intergenic region between *gspC* and *gspG*, which proves direct activation of *gsp* gene expression by QsmR (Fig. 6).

VI. Expression analysis of genes encoding QS-dependent proteins by real-time RT-PCR.

To determine if the genes encoding QS-dependent proteins were regulated by QS at transcriptional level, we performed real-time RT-PCR to compare gene expression levels in the wild type and the *tofI* mutant. The genes encoding flagellin (GR1, GR2, GR3, GR4, and GR20), a lipase precursor (GR15 and GR22), Trp-1 (GR34, RC5, and RC16), and hypothetical proteins (GR23, GR27, RC3, RC4, and RC14) that have homologies with *B. thalandensis* FlgE (YP_440801) which are already known as being QS-regulated were not analyzed by real-time RT-PCR. Of the 64 QS-upregulated proteins, expression of the corresponding genes for 57 of these proteins was activated by QS at the transcriptional level (Tables 3-6). There are also disagreement between the proteomic analysis and results of the real-time RT-PCR. Among the 46 extracellular QS-upregulated proteins, the expression patterns of the genes encoding 34 extracellular proteins were concored with our proteomics data (Table 3). However, the gene expression of the QS-upregulated DnaK (GR43) chaperone protein and hypothetical protein (GR45) was higher in the *tofI* mutant than in the wild type (Table 3). The expression level of the genes corresponding to the QS-downregulated extracellular proteins appeared to be higher in the wild type than the *tofI* mutant exept DnaK (GS1), protease Do

(GS3), and translation elongation factor Ts (GS4) genes (Table 4). Gene expression levels of QS-dependent cellular proteins matched with the results of real-time RT-PCR, exept the gene encoding hypothetical proteins RC7, RC10, SC1 and SC2 (Tables 5 and 6).

VII. Virulence of T2SS-deficient mutants.

To determine the effects of the *gsp* genes on *B. glumae* virulence, strains containing individual mutations in each *gsp* gene were inoculated into rice panicles at the flowering stage. The wild type caused severe grain rot, whereas the *gsp* mutant strains exhibited much less virulence in rice panicles. When pPW2 carrying *gsp* genes was introduced into the *gsp* mutant strains, the disease severity increased to that of the wild type (Fig. 7). These results indicated that the T2SS is important for full virulence in *B. glumae*.

DISCUSSION

Several proteomic analyses of *Burkholderia pseudomallei*, *B. thailandensis*, *B. cepacia*, and *B. cenocepacia* provide a reference map to identify the potential factors for virulence, stress tolerance, and viability (Riedel *et al*., 2003; Riedel *et al*., 2006; Wongtrakoongate *et al*., 2007; Thongboonkerd *et al*., 2007; Osiriphun *et al*., 2009). However, QS-dependent proteomic analysis has only been reported in the opportunistic pathogen *B. cepacia*, in which 55 proteins out of 985 detected spots were differentially expressed (Riedel *et al*., 2003). Due to missing genome data at that time only limited number of proteins could be identified by N-terminal sequencing (Riedel *et al*., 2003). Therefore, we reported on the first proteomic analysis of QS-dependent proteins in a member of the genus *Burkholderia* based on whole genome information. Comparing the identified QS-regulted proteins of *B. cepacia* with those of *B. glumae*, there were surprisingly no common proteins or protein homologues in the list. Because it seems that the imcompleteness of proteomic analysis in *B.* cepacia is caused by the lack of whole genome information. In addition, it is likely due to the different hosts of the two pathogens. Considering that the ecological niches these pathogens inhabit are quite different, QS may play different roles for each bacterium to take advantages in their environment.

In *P. aeruginosa*, proteomic analyses of QS-dependent protiens have

been carried out (Arevalo-Ferro *et al*., 2003; Nouwens *et al*., 2003). However, the identified QS-regulted proteins in *P. aeruginosa* are completely different from those in *B. glumae*, exept the flagellin protein. Since *B. glumae* and *P. aeruginosa* utilize different kinds of HSLs for QS, it is not surprising that they have different QS regulon. Additionally, it is not reasonable to compare the previously reported QS-dependent proteomes of *P. aeruginosa* with those of *B. glumae* identified in this study because our proteomic analysis was performed based on a single time point (exponential phase). The different proteome profiles between the wild type and the *tofI* mutant were not due to any growth differences of the two strains because no growth differences were observed. This indicates that QS regulates genes depending upon bacterial growth stages and reflects the broad and global gene regulation by QS depending upon the species of bacteria and their signaling molecules.

One common phenomenon between QS-regulated protein in *P. aeruginosa* and *B. glumae* is that the number of extracellular proteins is significantly reduced in the QS-deficient mutants (Nouwens *et al*., 2003). This is due to regulation of T2SS genes by QS in *P. aeruginosa* (Chapon-Herve *et al*., 1997). Likewise, the reduced amount of extracellular proteins in the *B. glumae* QS mutant was caused by significantly reduced expression of T2SS genes in this QS mutant. In *P. aeruginosa*, expression of *xcp* genes is depedent upon

LasR and RhlR. However, it is not clear which transcriptional regulator directly regulates *xcp* gene expression because the LasR/3-oxo-C12-HSL and RhlR/C4-HSL regulatory circuits are interdependent (Chapon-Herve *et al*., 1997). In this study, we clearly demonstrated that QsmR, a member of the TofR regulon, directrly regulates *gsp* gene expression in *B. glumae*. Besides, the regulation of T2SS gene expression by QS is not limited to these two bacteria. There is an indication that expression of *out* genes (T2SS genes) in Erwinia carotovora may be regulated in a cell density-dependent manner because expression of *out* genes is greatly increased in the early stationary phase (Sandkvist *et al*., 2001). However, there are no direct evidences about whether QS actually regulates expression of the *out* genes in *E. carotovora*.

Since QS regulated T2SS genes in *B. glumae*, it is important to determine if the genes corresponding to the identified QS-dependent extracellular proteins are directly regulated by QS-dependency of those extracellular proteins is indirect due to up-regulation of T2SS by QS. The previous studies do not distinguish proteins that are directly controlled by QS from T2SS-dependent proteins. The fact that the number of extracellular proteins is greatly reduced in the *P. aeruginosa* and *B. glumae* QS mutants raises the possibility that many extracellular proteins that appear to be QS-dependent are not actually controlled by QS but decrease due to direct

regulation of T2SS genes by QS. In fact, expression of large numbers of the genes encoding QS-dependent proteins is actually regulated by QS. For example, reduction of the concentration of LasB in the culture supernatant of the QS-deficient mutant of *P. aeruginosa* is correlated with reduced gene expression of *lasB* (Winson *et al*., 1995; Nouwens *et al*., 2003; Pearson *et al*., 1997).

In *B. glumae*, there were two proteins for which the corresponding gene and protein expression patterns were not correlated among the tested proteins in group I. According to the results of real-time RT-PCR, expression of the gene encoding GR32, an extracellular protein only found in the wild type, is reduced in the wild type. Although GR32 appears to be T2SS-dependent, it is predicted to possess no signal peptide and it might be secreted via alternative secretory pathways. The other protein, GR45, is predicted to have a signal peptide, and is secreted via the T2SS, thus, it is not clear why its gene expression level is reduced in the wild type. The discrepancy between 2-DE analysis and real time RT-PCR results of GR32 and GR34 is not caused by growth stage or growth conditions because all cells were harvested from the same culture condition. Further work will be required to answer this question in detail. Since QS regulates gene expression of the T2SS and T2SS substrates, this dual regulation should save energy and prevent premature secretion of proteins until the

bacteria have reached a critical cell density.

The extracellular proteins in group II were positively regulated by QS at the transcriptional level, except DnaK (GS1), protease Do (GS3), and translation elongation factor Ts (GS4). However, this sort of discrepancy is common in QS-dependent proteomic analyses (Arevalo-Ferro *et al*., 2003). One plausible explanation for this phenomenon is that QS-regulated extracellular proteases in the wild type but not in the *tofI* mutant degrade some of these proteins during our protein preparation procedure. Thus, the intensity of protease-sensitive protein spots would decrease in the wild-type samples. Besides, we cannot rule out the possibility that QS might regulate these proteins at a translation level.

It is somewhat contradictory finding that DnaK (GR32 and GS1) was detected as a secreted protein at elevated levels in both the wild type and in the *tofI* mutant, eventhough expression of the corresponding gene was negatively regulated by QS. The size differences between GR32 and GS1 may be a result of differential processing of DnaK production isoforms of difference lengths. However, there is inconclusive evidence for this phenomenon, and further work will be required to clarify this finding.

Among the *B. glumae* QS-regulated extracellular proteins in group I, 16 are secreted via the T2SS depite having no signal peptide. It has been reported

that cytoplasmic proteins such as EF-Tu and DnaK that have no signal peptide are secreted mostly through the mechano-sensitive MscL channel into the periplasm during osmotic down-shock or at low nutrient concentrations (Berrier *et al*., 2000). Therefore, it is possible that these proteins are released from the periplasm to the culture supernatant via T2SS by the mechano-sensitive mechanism in *B. glumae*. These 16 proteins could also use an alternative but previously unidentified secretion pathway.

The T2SS is known to be important in host-pathogen interactions (Jha *et al*., 2005). T2SS-dependent secretory proteins are involved in host tissue degradation, host colonization and the induction of plant defese responses by producing phytoalexin, oxidative bursts, and hypersensitive response-like effects (Jha *et al*., 2005). The *out* gene mutants of *E. chrysanthemi* are unable to secrete plant cell wall-degrading enzymes and are thus avirulent on African satiba (*Saintpaulia ionantha*) (Andro *et al*., 1984). Lipase breaks down epicuticular waxes (which cover host epidermal cells) and xylan (a plant cell wall component) (Devescovi *et al*., 2007) and this protein in *Xanthomonas oryzae* pv. *oryzae* was reported to be involved in pathogenicity as the plant cell wall degrading enzyme (Rajeshwari *et al*., 2005). Because the T2SS mutants of *B. glumae* were less virulent than the wild-type, we hypothesize that the T2SS-dependent virulence factors known in other bacterial pathogens (e.g.,

proteases, lipases, phosphatases, and elongation factor Tu) might play critical roles in the virulence of *B. glumae*. Indeed, it has been reported that *B. glumae* lipase is important for virulence (Devescovi *et al*., 2007).

Some other proteins that are known to be important for virulence in various pathogens are also controlled by QS in *B. glumae*. These include PAMPs (elongation factor Tu, DnaK, and trigger factor), serine metalloproteases, filamentous hemagglutinins, Hcp, enzymes for degrading aromatics and chloroaromatics, and hypothetical proteins that have enzymatic activity or unknown functions. PAMPs play key roles in triggering the innate immune responses in host cells (Jones *et al*., 2006; Zipfel *et al*., 2006). Secreted proteases that may be involved in virulence have been reported from bacterial pathogens, including *B. pseudomallei* (Gauthier et al., 2000). Expression of a novel *B. pseudomallei* serine metalloprotease is controlled by QS, but it plays minor role in virulence, which is unlike its contribution to the pathogenesis of *B. cenocepacia* respiratory infections (Valade et al., 2004; Sokol et al., 2003). Further, it is well known that filamentous hemagglutinins, Hcp, and enzymes for the degradation of aromatics and chloroaromatics respectively contribute to bacterial attachment to the host (Inatsuka *et al*., 2005), colonization (Mougous *et al*., 2006; Filloux *et al*., 2008), and survival of pathogenic bacteria (Gross *et al*., 2008; Reineke *et al*., 2001). Thus, even though no detailed studies on T6SS,

filamentous hemagglutinins, Hcp, or aromatic and chloroaromatic compound degrading enzymes have been performed in *B. glumae*, it is very likely that the identified, QS-dependent homologs of these proteins may have important roles in the interaction of *B. glumae* with rice cells.

This is the first report of the QS regulons of *B. glumae* derived via a proteomics approach. Considering that there are two major transcriptional regulators, TofR and QsmR, our proteomic analysis of the QS regulon did not address the issue of which proteins belong to which regulon. Further global analyses (e.g., transcriptomics and proteomics) concerning the QsmR regulon, which are dependent on growth phase, would be helpful to further elucidate the QS-mediated gene regulation in *B. glumae*. Characterization of the biochemical functions of proteins that may be important for virulence will also be the focus of future studies.

LITTERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403-410.

Andro T, Chambost JP, Kotoujansky A, Cattaneo J, Bertheau Y, Barras F, Van Gijsegem F, Coleno A (1984) Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *J Bacteriol* 160:1199-1203.

Arevalo-Ferro C, Hentzer M, Reil G, Gorg A, Kjelleberg S, Givskov M, Riedel K, Eberl L (2003) Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol* 5:1350-1369.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340:783-795.

Berrier C, Garrigues A, Richarme G, Ghazi A (2000) Elongation factor Tu and DnaK are transferred from the cytoplasm to the periplasm of *Escherichia coli* during osmotic downshock presumably via the mechanosensitive channel mscL. *J Bacteriol* 182:248-251.

Bonas U, Stall RE, Staskawicz B (1989) Genetic and structural characterization of the avirulence gene avrBs3 from *Xanthomonas campestris*

pv. *vesicatoria*. *Mol Gen Genet* 218:127-136.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.

Chapon-Herve V, Akrim M, Latifi A, Williams P, Lazdunski A, Bally M (1997) Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. *Mol Microbiol* 24:1169-1178.

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

Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. *Annu Rev Microbiol* 61:401-422.

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.

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans.

Proc Natl Acad Sci USA 76:1648-1652.

Filloux A, Hachani A, Bleves S (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology* 154:1570-1583.

Gauthier YP, Thibault FM, Paucod JC, Vidal DR (2000) Protease production by *Burkholderia pseudomallei* and virulence in mice. *Acta Trop* 74:215-220.

Gorg A, Boguth G, Obermaier C, Posch A, Weiss W (1995) Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. *Electrophoresis* 16:1079-1086.

Gross R, Guzman CA, Sebaihia M, dos Santos VA, Pieper DH, Koebnik R, Lechner M, Bartels D, Buhrmester J, Choudhuri JV, Ebensen T, Gaigalat L, Herrmann S, Khachane AN, Larisch C, Link S, Linke B, Meyer F, Mormann S, Nakunst D, Ruckert C, Schneiker-Bekel S, Schulze K, Vorholter FJ, Yevsa T, Engle JT, Goldman WE, Puhler A, Gobel UB, Goesmann A, Blocker H, Kaiser O, Martinez-Arias R (2008) The missing link: *Bordetella petrii* is endowed with both the metabolic versatility of environmental bacteria and

virulence traits of pathogenic *Bordetellae*. *BMC Genomics* 9:449.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720-1730.

Huynh TV, Dahlbeck D, Staskawicz BJ (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* 245:1374-1377.

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

Inatsuka CS, Julio SM, Cotter PA (2005) Bordetella filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity. *Proc Natl Acad Sci USA* 102:18578-18583.

Jefferson R A, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901-3907.

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

Jha G, Rajeshwari R, Sonti RV (2005) Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. *Mol Plant-Microbe Interact* 18:891-898.

Jones JD, Dangl JL (2006) The plant immune system. *Nature* 444:323-329.

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.

Khajanchi BK, Sha J, Kozlova EV, Erova TE, Suarez G, Sierra JC, Popov VL, Horneman AJ, Chopra AK (2009) *N*-acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and *in vivo* virulence in a clinical isolate of *Aeromonas hydrophila*. *Microbiology* 155:3518-3351.

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.

Lim J, Lee TH, Nahm BH, Choi YD, Kim M, Hwang I (2009) Complete genome sequence of *Burkholderia glumae* BGR1. *J Bacteriol* 191:3758-3759.

Liu H, Coulthurst SJ, Pritchard L, Hedley PE, Ravensdale M, Humphris S, Burr T, Takle G, Brurberg MB, Birch PR, Salmond GP, Toth IK (2008) Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLoS Pathog* 4:e1000093.

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.

Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordonez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526-1530.

Nouwens AS, Beatson SA, Whitchurch CB, Walsh BJ, Schweizer HP, Mattick JS, Cordwell SJ (2003) Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149:1311-1322.

Osiriphun Y, Wongtrakoongate P, Sanongkiet S, Suriyaphol P, Thongboonkerd V, Tungpradabkul S (2009) Identification and characterization of RpoS regulon and RpoS-dependent promoters in *Burkholderia pseudomallei*. *J Proteome Res* 8:3118-3131.

Parker CT, Sperandio V (2009) Cell-to-cell signalling during pathogenesis. *Cell Microbiol* 11:363-369.

Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260:1127-1130.

Pearson JP, Pesci EC, Iglewski BH (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* 179:5756-5767.

Rajeshwari R, Jha G, Sonti RV (2005) Role of an in planta-expressed xylanase of *Xanthomonas oryzae* pv. *oryzae* in promoting virulence on rice. *Mol Plant-Microbe Interact* 18:830-837.

Reineke W (2001) Aerobic and anaerobic biodegradation potentials of microorganisms. *The Handbook of Environmental Chemistry* 2K:1-161

Riedel K, Arevalo-Ferro C, Reil G, Gorg A, Lottspeich F, Eberl L (2003) Analysis of the quorum-sensing regulon of the opportunistic pathogen

Burkholderia cepacia H111 by proteomics. *Electrophoresis* 24:740-750.

Riedel K, Carranza P, Gehrig P, Potthast F, Eberl L (2006) Towards the proteome of *Burkholderia cenocepacia* H111: setting up a 2-DE reference map. *Proteomics* 6:207-216.

Robert V, Filloux A, Michel GP (2005) Role of XcpP in the functionality of the *Pseudomonas aeruginosa* secreton. *Res Microbiol* 156:880-886.

Ruvkun GB, Ausubel FM (1981) A general method for site-directed mutagenesis in prokaryotes. *Nature* 289:85-88.

Sambrook J, Russell DW (2001) *Molecular Cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor, NY.

Sandkvist M (2001) Type II secretion and pathogenesis. *Infect Immun* 69:3523-3535.

Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185:2066-2079.

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* 149:3649-3658.

Staskawicz B, Dahlbeck D, Keen N, Napoli C (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* 169:5789-5794.

Subsin B, Chambers CE, Visser MB, Sokol PA (2007) Identification of genes regulated by the *cepIR* quorum-sensing system in *Burkholderia cenocepacia* by high-throughput screening of a random promoter library. *J Bacteriol* 189:968-979.

Thongboonkerd V, Vanaporn M, Songtawee N, Kanlaya R, Sinchaikul S, Chen ST, Easton A, Chu K, Bancroft GJ, Korbsrisate S (2007) Altered proteome in *Burkholderia pseudomallei rpoE* operon knockout mutant: insights into mechanisms of *rpoE* operon in stress tolerance, survival, and virulence. *J Proteome Res* 6:1334-1341.

Ulrich RL, Deshazer D, Brueggemann EE, Hines HB, Oyston PC, Jeddeloh JA (2004) Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *J Med Microbiol* 53:1053-1064.

Valade E, Thibault FM, Gauthier YP, Palencia M, Popoff MY, Vidal DR (2004) The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. *J*

Bacteriol 186:2288-2294.

von Bodman SB, Willey JM, Diggle SP (2008) Cell-cell communication in bacteria: united we stand. *J Bacteriol* 190:4377-4391.

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185:2080-2095.

Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC (2001) Quorum-sensing in gram-negative bacteria. *FEMS Microbiol Rev* 25:365–404.

Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW, Lazdunski A, Stewart GSAB, Williams P (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92:9427-9431.

Wongtrakoongate P, Mongkoldhumrongkul N, Chaijan S, Kamchonwongpaisan S, Tungpradabkul S (2007) Comparative proteomic profiles and the potential markers between *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *Mol Cell Probes* 21:81-91.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix

G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125:749-760.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
Escherichia coli		
DH5α	F$^-$ Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* ($r_K^- m_K^+$) *deoR thi-1 supE44 λ-gyrA96 relA1*	Gibco BRL
S17-1	Tra+, *recA*, Sp^R	
C2110	*polA*, Nal^R	
BL21(DE3)	F$^-$*ompT hsdS*$_B$($r_B^- m_B^-$)*gal dcm* (DE3)	Novagen
Burkholderia glumae		
BGR1	Wild-type, Rif^R	Jeong *et al*. (2003)
BGS2	BGR1 *tofI*::Ω	Kim *et al*. (2004)
BGS9	BGR1 *qsmR*::Ω	Kim *et al*. (2007)
BGPW2	BGR1 gspD::Ω	Kang *et al*. (2008)

Continued for following pages

Table 1 – *Continued*

BEG75/BSG75/BQG75	BGR1 *gspD*::Tn*3-gusA75*/BGS2 *gspD*::Tn*3-gusA75*/BGS9 *gspD*::Tn*3-gusA75*	This study
BEG85/BSG85/BQG85	BGR1 *gspE*::Tn*3-gusA85*/BGS2 *gspE*::Tn*3-gusA85*/BGS9 *gspE*::Tn*3-gusA85*	This study
BEG57/BSG57/BQG57	BGR1 *gspF*::Tn*3-gusA57*/BGS2 *gspF*::Tn*3-gusA57*/BGS9 *gspF*::Tn*3-gusA57*	This study
BEG3/BSG3/BQG3	BGR1 *gspC*::Tn*3-gusA3*/BGS2 *gspC*::Tn*3-gusA3*/BGS9 *gspC*::Tn*3-gusA3*	This study
BEG60/BSG60/BQG60	BGR1 *gspG*::Tn*3-gusA60*/BGS2 *gspG*::Tn*3-gusA60*/BGS9 *gspG*::Tn*3-gusA60*	This study
BEG352/BSG352/BQG352	BGR1 *gspH*::Tn*3-gusA352*/BGS2 *gspH*::Tn*3-gusA352*/BGS9 *gspH*::Tn*3-gusA352*	This study
BEG17/BSG17/BQG17	BGR1 *gspJ*::Tn*3-gusA17*/BGS2 *gspJ*::Tn*3-gusA17*/BGS9 *gspJ*::Tn*3-gusA17*	This study
BEG139/BSG139/BQG139	BGR1 *gspK*::Tn*3-gusA139*/BGS2 *gspK*::Tn*3-gusA139*/BGS9 *gspK*::Tn*3-gusA139*	This study
BEG80/BSG80/BQG80	BGR1 *gspL*::Tn*3-gusA80*/BGS2 *gspL*::Tn*3-gusA80*/BGS9 *gspL*::Tn*3-gusA80*	This study
BEG33/BSG33/BQG33	BGR1 *gspM*::Tn*3-gusA33*/BGS2 *gspM*::Tn*3-gusA33*/BGS9 *gspM*::Tn*3-gusA33*	This study
BEG193/BSG193/BQG193	BGR1 *gspN*::Tn*3-gusA193*/BGS2 *gspN*::Tn*3-gusA193*/BGS9 *gspN*::Tn*3-gusA193*	This study

Continued for following pages

Table 1 – *Continued*

Plasmids		
pLAFR3	Tra$^-$, Mob$^+$, RK2 replicon, Tet^R	Staskawicz *et al*. (1987)
pLAFR6	As pLAFR3 but without *lacZα*, contains multilinker of pUC18 flanked by synthetic *trp* terminators, Tet^R	Huynh *et al*. (1989)
pHoKmGus	Promotorless β-glucuronidase gene, Km^R, Amp^R	Bonas *et al*. (1989)
pSShe	Cm^R	Stachel *et al*. (1985)
pLysS	Encodes T7 lysozyme gene, Cm^R	Novagen
pQSMR-His	*qsmR* in pET21b, Amp^R	Kim *et al*. (2007)
pHP45Ω	Ω cassette, Sp^R, Sm^R	Pretki *et al*. (1984)
pBGA43	0.85kb *EcoR*V-*Sfi*I fragment including *tofI* from pBGA18 cloned into pLAFR3	Kim *et al*. (2004)
pPW2	20-kb DNA fragment from strain BGR1 cloned into pLAFR3	Kang *et al*. (2008)

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

Table 2. List of oligonucleotide primers used in this study.

primer	sequence (5'→3')	primer	sequence (5'→3')
GR6L	GCGAGCTTCAGCAACTACG	GR6R	TGTAGGACGCGTAACTGTCG
TU1-F	ATGGCAAAAGAGAAGTTTGAA	TU-R1	CGATTTCGTCGTACTTCTT
GR9E-F	CATGAAACATAAATCATTGG	GR9E-R	CATCCGGACTGGTATTGTTGTC
GR10L	CGGAGTTGAAGGAAGAGACG	GR10R	ACGGATCACGCCAATAAGG
GR11E-F	GTCATGCGTATTTCCGTTGT	GR11E-R	GAAACGTCGCAGCGGTTCCATAG
GR12L	ACCGTAGTAGACGGTGATGG	GR12R	CGAAGAACGTATTCACCAACG
GR13L	CGACAATGCCTTCAATTCG	GR13R	GGCGATCGAGGTGTAGAAGC
GR16E-F	GGGCGGCTACGTCAACAATC	GR16E-R	CCAGTAGTTCTGCCGATAGATC
GR17L	CGACTACTCGACCACCTACACC	GR17R	CGATGACAGGACGAACTGC
GR18L	ATCAAGAATACCGCCAATGC	GR18R	TCGCATTACTGCTGCTATGG
GR19L	CAGAACATCGGCGACTACC	GR19R	TGTGATAGAACAGCAGGTTCG
GR24L	ACTGCGTGAGTTCCTTCTCG	GR24R	GCTACGTTGCCTACGACACC
GR25E-F	GGAGAAGGCCTACCTGTATC	GR25E-R	CATCGGCTGCGTAAGCTGGTT
GR29E-F	ATCGACGCCGACCTCATCAA	GR29E-R	GCCGAGGATCGCGAGATTGA
GR31E-F	CGGAGCAACGACATGAAAGT	GR31E-R	CGTCCTTGAACGCATAGAAG
GR39E-F	GCGAAGACGGTCGGCAAGGACGGTC	GR39E-R	GCCAGGCGCGCCATGTTGAAGCT
GR40E-F	CCAACATCGACCTGGAAAAGAC	GR40E-R	CGCCCTTGTCCACCAGGTACTT
GR41E-F	AGGGCGGCACTGTCTACGTG	GR41E-R	GGGACCGGTGAAGTACCAAC
GR42EH-F	GGCTTCGGTGATATGTTCTG	GR42E-R	GCCGCGACGCAGATTCGCAT
GR44L	ATGTTGTTCGCCGAGAGC	GR44R	GTCGAATTGCATCCAGAGC
GR45E-F	GCGAAGGTCTATTTCGTCCG	GR45E-R	ACGACGTCGGTCTGATTCTT
GR46L	GAATACGAACTACACCGACACG	GR46R	ACCTTGCCGATCACTTGC
GS1L	TTCTGCACTTCCTTCTCTTCG	GS1R	GCGTACATGGACGACAACG
GS2E-F	CGATTGAAGCCATGGCTAAC	GS2E-R	CTTCGCGAGTTTCTGAATAC

Continued for following pages

Table 2 – *Continued*

primer	sequence (5'→3')	primer	sequence (5'→3')
GS3L	CCGTTCTACCAGTTCTTCAAGC	GS3R	GCGTTGGTAAGGATGTAGCC
GS4L	AATCGTCATTCTTGGCAACG	GS4R	TGAAGCTCGGCAACAAGG
GS5E-F	CCTGCGACGGCTTCTTCTAC	GS5E-R	GTGGATCACGGTGACCTTCT
GS6E-F	CAAAGGAAGACCCAAATGCCG	GS6E-R	GAAGTCCGCCGGATAGAACA
GS7L	CGAGTTGACCACCATCTGC	GS7R	CGAGGTCAACTACCTGAACG
GS8E-F	CGATGAGTTGGTATAACAAG	GS8E-R	GCCGAAACCCCAGAAAAACG
GS9E-F	CGACAGGAGAGAGGTTTTGG	GS9E-R	AGCGTCGACCATGCCATCTG
GS10E-F	CATGTCCGACGTCGAAAACC	GS10E-R	ACGTCGACTTCCACCTCAAC
GS11E-F	GTTGAACTGCATACGAACCA	GS11E-R	CCGGCCTTCACGTAGTTCAG
GS12E-F	GTCGATGCGTCATGTTAGAT	GS12E-R	GACCAGTCCCATGACTTGAG
RC1EH-F	GCCATCGACCATTTCCGCTA	RC1EH-R	GAAGTTCCACGGAATGATCT
RC2L	GTGCCGATGGTGAAGAAGC	RC2R	TCGTCGTAGAACTTCAGATGC
RC6E-F	CGACATCAACACCCAATTCG	RC6E-R	TCATGTACAGCTCTTTCGAG
RC7E-F	TGCTTAACCATGGCGAAAAC	RC7E-R	GCCGTAGCTGAAGGTCTTCA
RC8L	AGAACATCACCTGCATCAGC	RC8R	GCTCGAACTCCTTGTTCTCG
RC12E-F	CGGCCGGCTACGACAATCAG	RC12E-R	TCGGACGAGGAAGTCACCAC
RC15L	GCATACTCGCTGCTGTTCC	RC15R	GTCGTCGAGTTCCATCACG
RC17E-F	CGATGACAACGAGGTGTTTG	RC17E-R	GACGGTGATGAACTGGAACT
RC18E-F	CAGCGAGCTGGTGTTTCATC	RC18E-R	TCGTCGCCGACGAGTTTTGC
SC1E-F	CTCGTTCTACGAATTCATCAC	SC1E-R	GTTCGCGGTCATGCGGAAGA
SC2E-F	GGAATCATCATGGAACTGAG	SC2E-R	TCCTCCAGCGGCAACCGTTG
GR11c	CCAAAAAATTTCAACAAAGG	GR25c	GCAAGGCCTCGTTTATCGAC
GR39c	ACGCCGGCCTTCGAGGCGGAATA	GR40c	GATCCACGGTGCATCCACGATT

Continued for following pages

Table 2 – *Continued*

primer	sequence (5'→3')	primer	sequence (5'→3')
GR41c	GCCGGGAAATCAGATGTTGA	GS8c	GCGGTTTCCATTTACCAATC
GS9c	CTATAATGGCCGTTTCGTAAAAT	GS11c	GTTTCACCCACCTCTAAAGAAG
RC6c	TGCTTCCTCCATCATCTAAG	RC7c	CGAAACCCGGAGCGCTGCTTAACCA
RC18c	CGACGGCGAGATTCATCTGA	SC2c	CTGCCGCGCTCATCTCATCC
gspDE-F	ATATCGCACCGACAACAACC	gspDE-R	GATCCAGACTTCTAGCGCAT
gspEF-F	AGATCCACAACAACGCGCCCGACT	gspEF-R	GTCTGCCTCGATCACGCCCTTCT
gspGH-F	TATCTCGAGCGCCTGCCGAAC	gspGH-R	AGCATCTCGAGCAGCGTGAAG
gspLM-F	TTCAAGCCGCAGGTCAAGGTC	gspLM-R	CAGCACCGAATAGAGCACCA
gspMN-F	AAGCAGTTCAAGGTACAGGTG	gspMN-R	GGGTCGACCAGGTTGAGGAG
gspHI-F	TCTATCGCCCGCGTGACTGG	gspHI-R	GATCAGCACCTCGATCATCG
gsgIJ-F	AGCAGCAGTTCGACTGTTCG	gspIJ-R	ATCTCGATCAGCGTGAAGC
gspJK-F	AAGCAAAACCAGGCGCTCAG	gspJK-R	AGCATGCCCGACACGAGGA
gspKL-F	ACAGCTGGCGATGCAGTCCA	gspKL-R	TCTTGTCGAGCAGCGCGAAC
gspF-R	CATCAGCTCGCGGATGTAGT	gspN-R	AGGGACCAGAACGAAGTCTG
gspDp-F	TATCATACCGGTGCCTCGTCGCATTGC	gspDp-R	AGCAAAACGAATGGTTGTCATGAG
gspCGp-F	GCGGGCTATTGTACGGATGA	gspCGp-R	GCATGAGTGAAACCTCTCTT
KEN1	ATCGGCCAAACGCAGCA	KEN2	AACGGCGGCATCAGCAA

Table 3. Identification of the extracellular proteins displaying greater than a 2.0-fold reduction in intensity in the QS mutant BGS2 relative to the wild-type BGR1.

Spot number	Protein description[a] [*B. glumae*]	Accession number/ gene ID	Matching sequence[b]	Observed migration[c]		Theoretical migration		Fold change[d]			Type II secretion		Real-time RT-PCR Fold change[h]	
				M_r (kDa)	p*I*	M_r (kDa)	p*I*	Mean	SD	*p* value[e]	Signal peptide[f]	2-DE[g]	Mean	SD
GR1	Flagellin	YP_002908906/ bglu_1g01710	**INSAADDAAGLAIATR**	42	4.4	39	4.8	31.35	4.1	<0.001	N	N	NT[i]	
GR2	Flagellin	YP_002908906/ bglu_1g01710	**SAADDAAGLTLSSSDQA**	42	4.5-4.6	39	4.8	17.91	4.96	0.002	N	N	NT	
GR3	Flagellin	YP_002908906/ bglu_1g01710	**TNLSSAQSIQSADFAQETAALSK**	42	4.7-4.8	39	4.8	9.97	1.06	<0.001	N	N	NT	
GR4	Flagellin	YP_002908906/ bglu_1g01710	**SAADDAAGLTLSSSDQATNLSSAIQSADFAQETAALSK**	42	4.9	39	4.8	6.86	1.38	<0.001	N	N	NT	
GR5	Serine metalloprotease	YP_002911503/ bglu_1g16590	**WAAGLSVVLNFSLGGGGSAPGVNILSTLNS**	42	6.2	39	6	19.81	6.85	0.023	27 and 28: AQA-QT	Y	7.16	1.9
GR6	Serine metalloprotease	YP_002911503/ bglu_1g16590	**APGVNILSTLNS**	42	6.6	39	6	7	2.32	0.033	27 and 28: AQA-QT	Y	7.16	1.9
GR7	Expressed protein GR12	YP_002909284/ bglu_2g17085	**FTNDGQSDVGGFPNAIMVQVS**	15	5.1	18	6.7	3.19	0.96	<0.001	14 and 15: DQR-SD	Y	59.37	16.94
GR8	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**LIAPIAME**	28	5.4	43	5.4	6.11	1.44	0.02	N	Y	2.01	0.41
GR9	Hypothetical protein	YP_002907540/ bglu_2p0150	**WPDNNTPNGMADGDIKPVYVR**	28	6.4	25	6.9	6.12	1.29	0.007	33 and 34: AYA-SY	Y	35.5	4.73

Continued for following pages

Table 3 - *Continued*

GR10	Expressed protein GR10	YP_002909132/ bglu_2g15435	**VPDVEGSPDPFVTPPTID**	20	5.4	18	5.2	3.64	0.65	0.035	N	N	7.21	3.17
GR11	Hypothetical protein	YP_002912410/ bglu_1g26310	**APYGTAATFQLAP**	15	4.2	15	8.9	13.15	4.26	0.011	43 and 44: AHA-AT	Y	2.9	0.58
GR12	Expressed protein GR12	YP_002909284/ bglu_2g17085	**NVFTNDGQSDVGGFAKPNA IMVQVSGPTDR**	15	4.8	18	6.7	>100		0.018	14 and 15: DQR-SD	Y	59.37	16.94
GR13	Secreted protein	YP_002911330/ bglu_1g14790	**TVGTAGSAASHANSLVVNA NDTLI**	53	6.2	64	7.4	>100		0.004	24 and 25: ALG-AC	Y	7.36	1.89
GR14	Secreted protein	YP_002911330/ bglu_1g14790	**TVGTAGSAASHANSLVVNA NDTLI**	53	6.4	64	7.4	>100		0.011	24 and 25: ALG-AC	Y	7.36	1.89
GR15	Lipase precursor (triacylglycerol lipase)	YP_002908426/ bglu_2g07730	**GSEFADFVQDVLK**	36	6.7	37	7.7	6.42	0.81	0.004	39 and 40: AVA-AD	Y	NT	
GR16	Hypothetical protein	YP_002910945/ bglu_1g10710	**GGETNFGITAATARTFEYG WQLNR**	21	6.2	20	5.9	>100		0.018	N	Y	12.91	0.68
GR17	Hypothetical protein	YP_002908411/ bglu_2g07580	**TTGSPWTLR**	49	4.5	53	4.3	>100		0.004	33 and 34: ASG-ST	Y	16.22	5.97
GR18	Filamentous hemagglutinin	YP_002909362/ bglu_2g17940	**TAQAGDLTLQSNGR**	40	4.5	315	4.8	>100		<0.001	21 and 22: AVA-ET	Y	3.04	0.91
GR19	Hypothetical protein	YP_002909324/ bglu_2g17490	**VYIYDAIFFDYFR**	44	5.1	52	6.6	14	4.06	<0.001	24 and 25: AFA-GA	Y	3.16	1.68
GR20	FliC	YP_002908537/ bglu_1g01710	**INSAADDAAGLAIATRSTLA TQAATGTLSSSDQAAAEK**	42	5.4	39	4.8	4.25	1.29	0.002	N	N	NT	
GR21	Serine metalloprotease precursor	YP_002911503/ bglu_1g16590	**VLNFSLGGGGSCSNILSTLN SGK**	38	5.5	39	6.0	>100		0.032	27 and 28: AQA-QT	Y	7.16	1.90

Continued for following pages

Table 3 - *Continued*

GR22	Lipase precursor (triacylglycerol lipase)	YP_002908426/ bglu_2g07730	**TLTTAQAAVYNRNAEDPLAVIR**	36	6.3	37	7.7	5.18	0.69	0.003	39 and 40: AVA-AD	Y	NT	
GR23	Hypothetical protein	YP_002913080/ bglu_1g33140	**VQAYDSLGNAK**	34	4.5	42	4.4	12.67	1.60	0.003	N	Y	NT	
GR24	Hypothetical protein	YP_002907869/ bglu_2g01460	**VLAVSAQLSPD**	33	4.6	50	7.1	>100		<0.001	N	Y	5.77	0.73
GR25	Adhesin HecA	YP_002908465/ bglu_2g08140	**SGTLAVNTQTL**	33	4.7	63	4.2	>100		0.002	40 and 41: VNT-QT	Y	6.10	0.14
GR26	Hypothetical protein	YP_002907869/ bglu_2g01460	**VLAVSAQLSPD**	33	4.7	50	7.1	>100		<0.001	N	Y	5.77	0.73
GR27	Hypothetical protein	YP_002913080/ bglu_1g33140	**FNLSTTDTAQTNS**	32	4.6	42	4.4	>100		0.018	N	Y	NT	
GR28	Hypothetical protein	YP_002909440/ bglu_2g18790	**VGLEDGLTVNDAR**	27	4.5	61	6.9	5.64	0.2	0.041	N	N	2.31	1.68
GR29	3-oxoadipate CoA-succinyl transferase beta subunit	YP_002911966/ bglu_1g21560	**VELADGVSIEEIK**	27	4.6	22	4.4	5.90	2.30	<0.001	N	N	16.41	0.25
GR30	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**LLDQGQAGDNVGILLR LIAPIAMEEGLR**	29	5	43	5.4	8.97	1.53	0.023	N	Y	2.01	0.41
GR31	ThiJ/PfpI domain protein	YP_002908191/ bglu_2g05090	**GFWLEELAA**	28	5.1	24	5.1	>100		0.056	N	Y	1.02	0.29
GR32	Chaperone protein DnaK	YP_002910531/ bglu_1g06340	**VLLLDVTPLSLGIETLGGV MTKLLGEFNLEGIPPAPR**	28	5.1	69	4.8	>100		0.0053	N	Y	0.38	0.25

Continued for following pages

Table 3 - *Continued*

GR33	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**LLDQGQAGDNVGILLRLIA PIAMEEGLR**	28	5.3	43	5.4	14.85	2.08	0.00227 0705	N	Y	2.01	0.41
GR34	Trp-1 (ToxA)	YP_002908311/ bglu_2g06400	**LVAYTVDPDFSLAKYGVNVL NER**	28	5.4	28	5.3	9.8	3.16	0.009	N	Y	NT	
GR35	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**LIAPIAMEEGLR**	28	5.3	43	5.4	>100		0.006	N	Y	2.01	0.41
GR36	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**LLDQGQAGDNVGILLRLIA PIAMEEGLR**	27	5.8	43	5.4	>100		0.044	N	Y	2.01	0.41
GR37	3-oxoacid CoA-transferase, A subunit	YP_002911967/ bglu_1g21570	**ITVAEVEEIVENGELDPDQI HTPGIFVQR**	29	5.9	49	11.5	7.89	3.15	0.012	N	N	2.86	0.58
GR38	Translation elongation factor Tu	YP_002910148/ bglu_1g02430	**AVDGTFLMPVEDVFSISGRL LDQGQAGDNVGILLRTTDV TGSIELPKLIAPIAMEEGLR**	27	6.1	43	5.4	>100		0.002	N	N	2.01	0.41
GR39	3-hydroxyacyl-CoA dehydrogenase	YP_002912680/ bglu_1g29130	**GNVFLITGGASGLGAGTAR**	29	6.3	26	6.1	>100		0.007	N	Y	1.88	0.47
GR40	Ankyrin repeat protein	YP_002911235/ bglu_1g13820	**NQVGVTALEFAK**	25	5	25	5.6	>100		0.024	36 and 37: AQA-ES	Y	5.97	0.19
GR41	Putative bacteriophage protein	YP_002911405/ bglu_1g15600	**AEVATIETQLIDPAAPPTAFG VAVK**	21	5.2	18	4.8	>100		0.013	N	N	1.68	0.39
GR42	Thiamine-phosphate pyrophosphorylase	YP_002910224/ bglu_1g03190	**LVVTSGDAE**	14	4.3	38	5.5	>100		0.007	N	Y	10.99	0.38
GR43	Hypothetical protein	YP_002910945/ bglu_1g10710	**GGETNFGITAATARTFEYG WQLNR**	22	5.5	20	5.9	>100		0.069	N	N	12.91	0.68

Continued for following pages

Table 3 - *Continued*

GR44	Hypothetical protein	YP_002912410/ bglu_1g26310	**AAPYGTAATFQLAPGR**	15	4.3	16	8.9	7.61	3.07	0.01	43 and 44: AHA-AT	Y	3.35	1.11
GR45	Hypothetical protein	YP_002912849/ bglu_1g30820	**LQAAWDQGNLADIR**	16	4.5	19	4.4	>100		0.001	14 and 15: GYA-AG	Y	0.46	0.25
GR46	NAD-dependent aldehyde dehydrogenase	YP_002912305/ bglu_1g25250	**DSGYGSEGGPEALEPYLVTK**	15	4.4	51	7.2	>100		0.039	N	Y	9.58	2.87

[a] Accession numbers and locus IDs were obtained from the *B. glumae* BGR1 genome database (Lim *et al*., 2009).

[b] In all sequences, L and Q represent the ambiguous pairs (I/L) and (K/Q). Amino acid differences are shown in bold.

[c] The M_r and p*I* values were estimated from the data in Fig. 2.

[d] The mean and standard deviation (SD) of the fold change was calculated by comparing spot intensities of the wild-type sample (*B. glumae* BGR1) to the autoinducer synthesis mutant (*B. glumae* BGS2) using quantitative image analysis software (PDQuest™ 2-D Analysis Software V 8.0).

[e] Student's *t*-test.

[f] The presence and location of the signal peptide cleavage sites in the primary sequences were predicted by the SignalP 3.0 server (Bendtsen *et al*., 2004); http://www.cbs.dtu.dk/services/SignalP). N = absence of signal peptide.

[g] Y = secretion of the protein found in 2-DE experiments. The spot was missing from the supernatant of the *gspD* mutant (type II secretion system mutant). N = the protein was not secreted through the T2SS.

[h] The mean and SD of the fold change was calculated by dividing the mean CT of BGR1 by that of BGS2.

[i] Not tested. These spots were previously described as QS up-regulated.

Table 4. Identification of the extracellular proteins displaying greater than a 2.0-fold increase in intensity in the QS mutant BGS2.

Spot number	Protein description[a] [*B. glumae*]	Accession number/ gene ID	Matching sequence[b]	Observed migration[c]		Theoretical migration		Fold change[d]			Real-time RT-PCR Fold change[h]	
				M_r (kDa)	p*I*	M_r (kDa)	p*I*	Mean	SD	*p* value[e]	Mean	SD
GS1	Chaperone protein DnaK	YP_002910531/ bglu_1g06340	**VSDIDDVILVGGQTR**	58	5	69	4.8	0.31	0.11	0.006	0.38	0.25
GS2	Trigger factor	YP_002911203/ bglu_1g13490	**IGQEFFEVSR** **IGDLATAEVER**	50	5	50	4.8	0.21	0.06	0.004	1.28	0.42
GS3	Protease Do	YP_002912733/ bglu_1g29660	**IDATGLPVVK** **GNSLALLIQR**	49	6.3	51	9.3	0.07	0.02	0.003	0.15	0.08
GS4	Translation elongation factor Ts	YP_002911134/ bglu_1g12730	**EVSLLNQPFVK** **FALFVVGEG**	36	5.4	31	5.3	<0.01		0.002	0.70	0.19
GS5	Thioredoxin-disulfide reductase	YP_002910699/ bglu_1g08120	**VLILGSGPAGYTAAVYAAR** **LAGNATATSVPGVFAA** **QAITSAGTGCMAALDAQR**	36	5.3	34	5.3	0.22	0.06	0.011	2.26	0.18
GS6	Alkyl hydroperoxide reductase/Thiol specific antioxidant	YP_002908962/ bglu_2g13670	**DVLIEEEGL** **RGTFVINPEGEIK**	23	4.5	21	4.9	<0.01		0.005	4.45	0.30
GS7	3-oxoacyl-(acyl carrier protein) synthase	YP_002910839/ bglu_1g09550	**NSFGFGGTNGTLVFK**	44	6	37	5.6	<0.01		0.014	1.39	0.44
GS8	Hypothetical protein	YP_002907869/ bglu_2g01460	**VLAVSAQLSPD**	35	4.3	50	7.1	<0.01		0.045	5.77	0.73
GS9	Hypothetical protein	YP_002910871/ bglu_1g09920	**EPSAAAMAAA**	26	4.9	31	12.2	<0.01		0.011	24.69	0.68

Continued for following pages

Table 4 - *Continued*

GS9	Hypothetical protein	YP_002910871/ bglu_1g09920	**EPSAAAMAAA**	26	4.9	31	12. 2	<0.01		0.011	24.69	0.68
GS10	Protein-export protein SecB	YP_002912943/ bglu_1g31760	**SDVENQPFFNIQR**	22	4.4	18	4.3	0.094	0.01	0.004	2.89	0.15
GS11	Peptidyl-prolyl cis-trans isomerase B	YP_002912154/ bglu_1g23650	**ENFLNYVKAG**	21	5.9	18	6.1	<0.01		0.050	19.13	0.17
GS12	Hemolysin-coregulated protein	YP_002910294/ bglu_1g03910	**EGLPVEQFSLK**	20	6.4	19	6.5	<0.01		0.014	3.15	0.78
GS13	Hemolysin-coregulated protein	YP_002910294/ bglu_1g03910	**YVIISSVTP QKEGLPVEQFSLK**	20	6.8	19	6.5	<0.01		0.009	3.15	0.78

[a-h] As in Table 3.

Table 5. Identification of cellular proteins displaying greater than a 2.0-fold reduction in intensity in the QS mutant BGS2.

Spot number	Protein description[a] [*B. glumae*]	Accession number/ gene ID	Matching sequence[b]	Observed migration[c]		Theoretical migration		Fold change[d]			Real-time RT-PCR Fold change[h]	
				M_r (kDa)	p*I*	M_r (kDa)	p*I*	Mean	SD	*p* value[e]	Mean	SD
RC1	Aldehyde dehydrogenase family protein	YP_002910002/ bglu_1g00950	**ALVEESIYER IFQEEIFGPVLSVTTFK**	47	6.2	55	6.3	>100		0.001	79.22	0.25
RC2	Hypothetical protein	YP_002909440/ bglu_2g18790	**LLAGNAQAATIR SPYLPITPEAI VGLEDGLTVNDAR**	47	6.8	59	6.9	>100		0.002	2.31	1.68
RC3	Hypothetical protein	YP_002908314/ bglu_2g06430	**YPVSNLEYR LPSEAEWEYAAAGGAAR**	38	5.4	35	5.1	>100		0.001	NT	
RC4	Hypothetical protein]	YP_002908314/ bglu_2g06430	**AYPGGNAIDDDLAVTQGAY R**	35	5.5	35	5.1	3.43	1.87	0.022	NT	
RC5	TRP-1	YP_002908311/ bglu_2g06400	**LVAYTVDPDFSLAK YGVNVLNER**	29	5.2	28	5.3	3.98	0.46	0.002	NT	
RC6	Hypothetical protein	YP_002908348/ bglu_2g06890	**TWAEILQNSQLAR**	29	5.5	28	5.4	>100		0.006	3.75	0.29
RC7	Hypothetical protein	YP_002912645/ bglu_1g28780	**LVYDELRR**	29	5.6	18	11.0	3.14	0.22	0.001	0.55	0.50
RC8	3-oxoacid CoA-transferase, A subunit	YP_002911967/ bglu_1g21570	**GIPAFFTNTGY**	29	5.9	25	5.5	5.177	1.53	0.003	2.86	0.58
RC9	3-oxoadipate CoA-succinyl transferase beta subunit	YP_002911966/ bglu_1g21560	**VELADGVSIEEIK**	28	4.4	22	4.5	>100		0.004	16.41	0.25

Continued for following pages

Table 5 - *Continued*

RC10	Hypothetical protein	YP_002912849/ bglu_1g30820	**LQAAWDQGNL**	28	4.6	36	10.6	5.54	0.47	0.026	0.46	0.25
RC11	Bacterial transferase hexapeptide repeat protein	YP_002909439/ bglu_2g18780	**LDADTSVWFGAVLREPIVIG EGTNVQDGAVLHTERGNIE FYADQR**	25	5.8	19	5.9	>100		0.003	2.08	0.58
RC12	WD-repeat protein	YP_002908313/ bglu_2g06420	**KVGEGVPEASGSTQLPPEV WPRS**	61	6.1	62	6.1	6.18	1.53	0.006	>100	0.53
RC13	3-oxoadipate CoA-succinyl transferase beta subunit	YP_002911966/ bglu_1g21560	**SGDLANWMIPGKM**	28	4.5	22	4.4	9.94	7.29	0.022	16.41	0.25
RC5	TRP-1	YP_002908311/ bglu_2g06400	**LVAYTVDPDFSLAK YGVNVLNER**	29	5.2	28	5.3	3.98	0.46	0.002	NT	
RC14	Hypothetical protein	YP_002908314/ bglu_2g06430	**RLFLEDTGSTELPSSWAFGV YPAERSREYPWGDAFDPAA ANTVEAGPLSTTPVGIFRAY**	38	5.2	35	5.1	6.10	1.16	0.019	NT	
RC15	Malate/lactate dehydrogenases	YP_002908471/ bglu_2g08210	**PGGNAIDDDLAVTQGAYRV KGVVMELDDCAFPLLAGV VITDDPKVKDLLSANAEIFT VQGAALNEVASRD**	38	5.6	34	5.4	14.21	9.21	0.056	3.51	0.81
RC16	TRP-1	YP_002908311/ bglu_2g06400	**RYDSIGGLFEDFTQSAAQRA RDVANMEPLGQFDLVNAA WLFNYADSVENLKSVLDLA CGFGFFGRE**	29	5.4	28	5.3	7.11	3.89	0.009	NT	
RC17	Two component transcriptional regulator, Fis family2	YP_002913144/ bglu_1g33780	**NHFLVIDDNEVF**	22	6.3	20	6.3	3.07	1.83	0.079	56.20	0.27
RC18	Hypothetical protein	YP_002911954/ bglu_1g21440	**ADGTLLVDLGR**	20	5.9	77	9.8	>100		0.031	3.45	0.33

[a-h] As in Table

Table 6. Identification of cellular proteins only found in the QS mutant BGS2.

Spot number	Protein description[a]	Accession number/gene ID	Matching sequence[b]	Observed migration[c]		Theoretical migration		Fold change[d]			Real-time RT-PCR Fold change[h]	
				M_r (kDa)	p*I*	M_r (kDa)	p*I*	Mean	SD	*p* value[e]	Mean	SD
SC1	Hypothetical protein	YP_002909437 / bglu_2g18760	**PGSFYEFITRDAGNAQGIFR**	40	6	38	5.9	<0.01		0.005	12.34	0.31
SC2	Hypothetical protein	YP_002912834 / bglu_1g30670	**ISLDQWQRIVPEAAPAWR**	25	6.1	19	6.0	<0.01		0.008	4.77	0.47

[a-h] As in Table 3.

Table 7. Expression of *gsp*::Tn*3-gusA* fusions in the *tofI* and *qsmR* mutant backgrounds.

Fusions	Activity of β-glucuronidase (10^{-11}U/colony forming unit/min)			
	BGR1 (Wild-type)	BGS2 (*tofI*::Ω)	BGS9 (*qsmR*::Ω)	BGS2(*tofI*::Ω) + 1μM C8-HSL
gspD::Tn*3-gusA75*	13.6 ± 0.1[a]	2.0 ± 0.1	2.2 ± 0.5	15.6 ± 0.2
gspE::Tn*3-gusA85*	16.4 ± 0.5	3.2 ± 0.8	4.4 ± 0.2	18.8 ± 0.3
gspF::Tn*3-gusA57*	15.2 ± 0.4	1.2 ± 0.6	1.6 ± 0.6	6.4 ± 0.4
gspC::Tn*3-gusA3*	8.8 ± 0.2	2.8 ± 0.2	2.4 ± 0.6	9.6 ± 0.1
gspG::Tn*3-gusA60*	87.6 ± 0.3	14.4 ± 0.2	22.0 ± 0.6	73.2 ± 0.3
gspH::Tn*3-gusA352*	6.8 ± 0.1	2.0 ± 0.3	2.0 ± 0.2	6.8 ± 0.4
gspJ::Tn*3-gusA17*	4.4 ± 0.5	1.2 ± 0.2	1.2 ± 0.2	5.6 ± 0.6
gspK::Tn*3-gusA139*	4.8 ± 0.2	1.2 ± 0.2	2.0 ± 0.7	4.4 ± 0.1
gspL::Tn*3-gusA80*	6.4 ± 0.1	1.2 ± 0.2	1.2 ± 0.4	7.2 ± 0.4
gspM::Tn*3-gusA33*	16.0 ± 0.4	3.6 ± 0.6	3.6 ± 0.5	16.8 ± 0.2
gspN::Tn*3-gusA193*	6.4 ± 0.4	1.2 ± 0.1	1.6 ± 0.6	6.8 ± 0.1

Figure 1. Classification of QS-dependent proteins. The 79 identified proteins in the *B. glumae* QS-dependent proteome were divided into two groups according to their localization. The groups were further divided into four subgroups based on comparative analysis of their intensities between the wild type BGR1 and QS mutant BGS2. Group I: extracellular proteins displaying greater than a 2.0-fold reduction in intensity in the *tofI* mutant relative to the wild-type; group II: extracellular proteins displaying greater than a 2.0-fold increase in intensity in the *tofI* mutant; group III: cellular proteins displaying greater than a 2.0-fold reduction in intensity in the *tofI* mutant; group IV: cellular proteins displaying greater than a 2.0-fold increase in intensity in the *tofI* mutant. Protein spots previously shown to be controlled by QS are denoted in the box along with the dotted line.

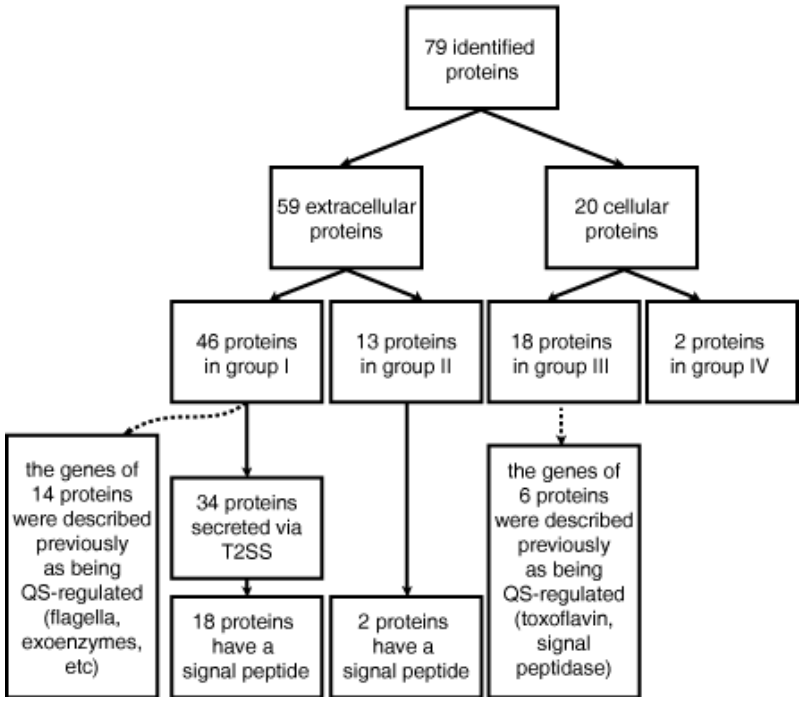
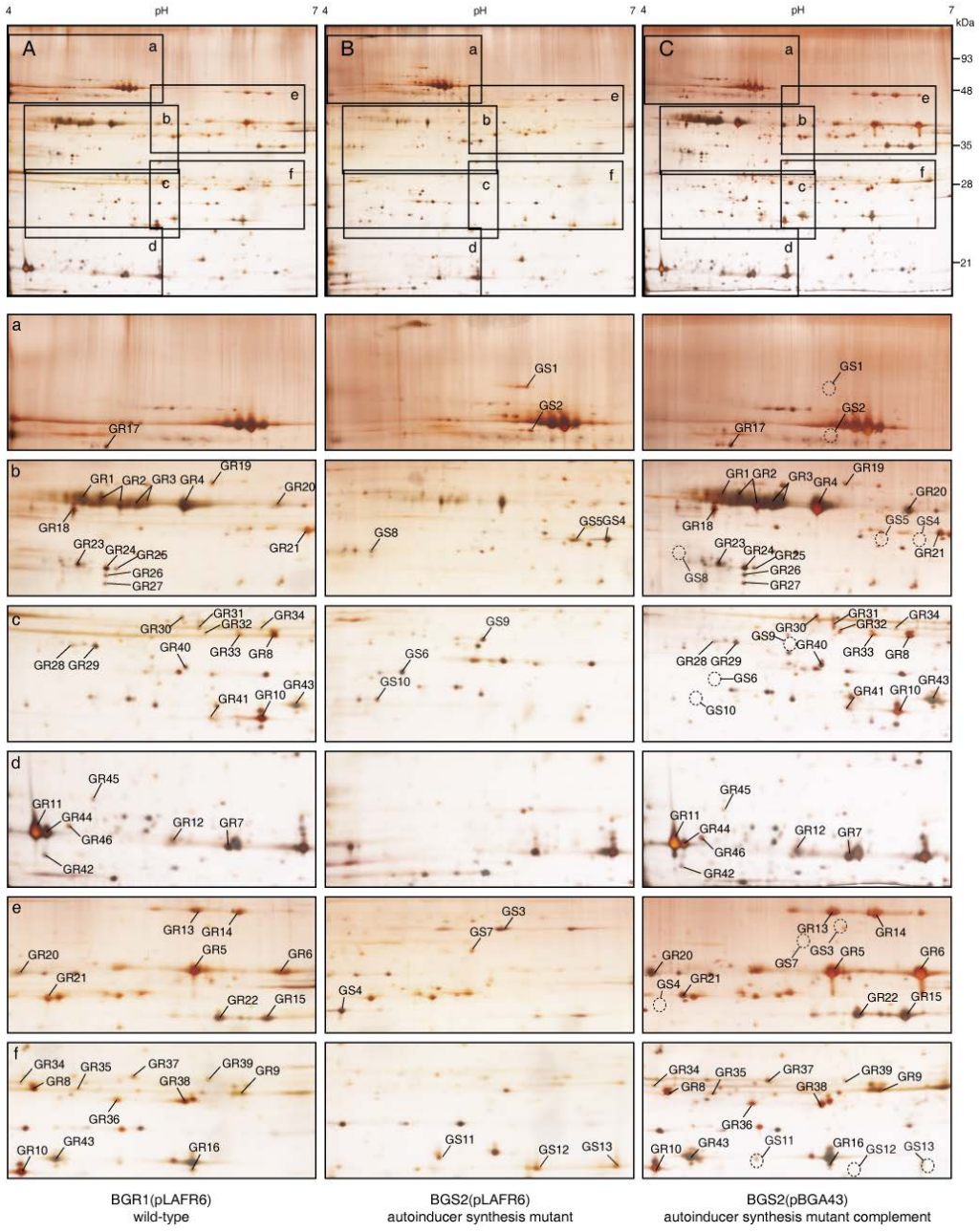

79 identified proteins
59 extracellular proteins
20 cellular proteins
46 proteins in group I
13 proteins in group II
18 proteins in group III
2 proteins in group IV
the genes of 14 proteins were described previously as being QS-regulated (flagella, exoenzymes, etc)
34 proteins secreted via T2SS
18 proteins have a signal peptide
2 proteins have a signal peptide
the genes of 6 proteins were described previously as being QS-regulated (toxoflavin, signal peptidase)

Figure 2. Comparative 2-DE of extracellular proteins of *B. glumae*. Strains BGR1(pLAFR6) (A), BGS2(pLAFR6) (B), and BGS2(pBGA43) (C) were grown in LB broth. The culture supernatants were harvested at early exponential phase, and 80 μg of protein from the supernatants was separated and stained with silver nitrate. The dotted circle in the third image signifies that the spots increased in intensity in the QS mutant BGS2. The numbers correspond to the spot numbers listed in Tables 1 and 2.



BGR1(pLAFR6)
wild-type

BGS2(pLAFR6)
autoinducer synthesis mutant

BGS2(pBGA43)
autoinducer synthesis mutant complement

Figure 3. Comparative 2-DE of cellular proteins in *B. glumae*. Strains BGR1(pLAFR6) (A), BGS2(pLAFR6) (B), and BGS2(pBGA43) (C) were grown in LB broth, and the cells were harvested at early exponential phase. A total of 80 μg of extracted protein was separated and stained with silver nitrate. The numbers correspond to the spot numbers listed in Tables 3 and 4.

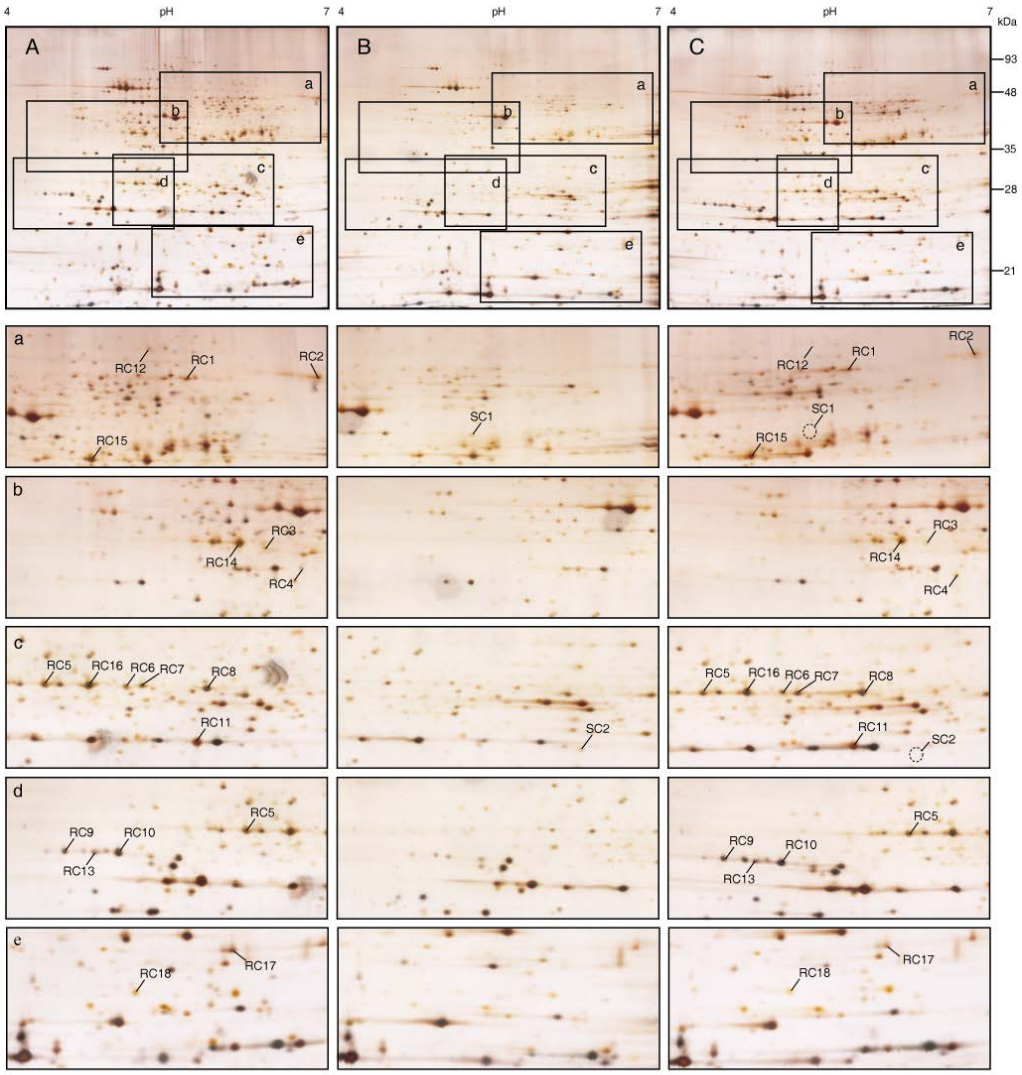

4
pH
7
kDa
93
48
35
28
21
A
B
C
a
b
c
d
e
RC12
RC1
RC2
RC15
SC1
RC3
RC14
RC4
RC5
RC16
RC6
RC7
RC8
RC11
SC2
RC9
RC10
RC13
RC17
RC18
BGR1(pLAFR6)
wild-type
BGS2(pLAFR6)
autoinducer synthesis mutant
BGS2(pBGA43)
autoinducer synthesis mutant complement

Figure 4. The 2-DE patterns of *B. glumae* proteins secreted through the T2SS in culture supernatants. Strains BGR1(pLAFR6) (A), BGPW2(pLAFR6) (B), and BGPW2(pPW2) (C) were grown in LB broth. The cultures were harvested at early exponential phase. A total of 80 μg of protein from the supernatants was separated and stained with silver nitrate. The numbers correspond to the spot numbers listed in Table 1.

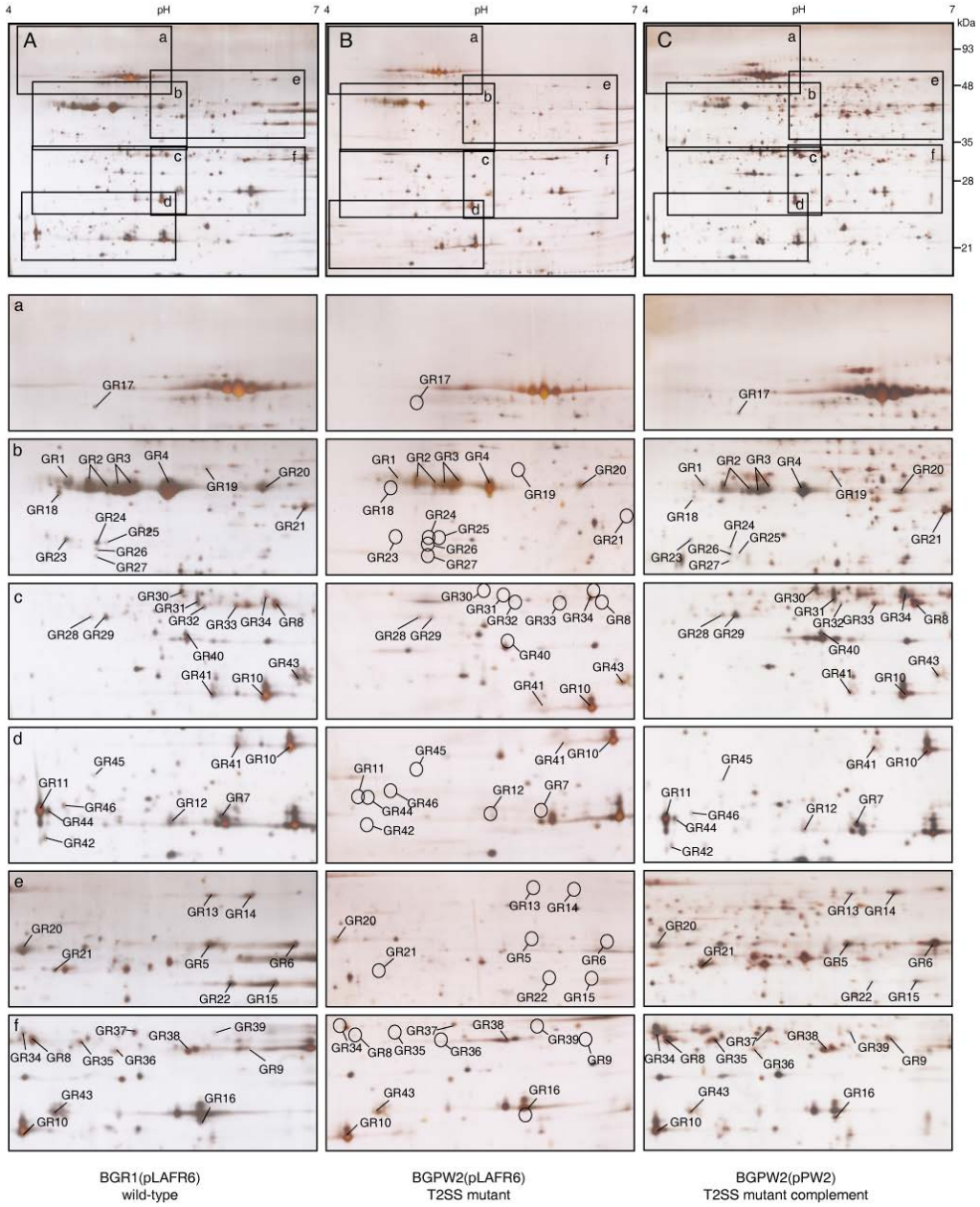

4
pH
7
A
B
C
a
b
c
d
e
f
kDa
93
48
35
28
21
GR17
GR1
GR2
GR3
GR4
GR19
GR20
GR18
GR24
GR25
GR21
GR23
GR26
GR27
GR30
GR31
GR32
GR33
GR34
GR8
GR28
GR29
GR40
GR43
GR41
GR10
GR45
GR11
GR46
GR12
GR7
GR44
GR42
GR13
GR14
GR5
GR6
GR22
GR15
GR37
GR38
GR39
GR35
GR36
GR9
BGR1(pLAFR6)
wild-type
BGPW2(pLAFR6)
T2SS mutant
BGPW2(pPW2)
T2SS mutant complement

Figure 5. (A) Genetic organization and restriction map of the position of the *gsp* genes in *B. glumae* BGR1. Large arrows indicate the positions and orientations of the *gsp* genes. Vertical bars on the map indicate the positions and orientations of the Tn*3-gusA* insertions. B, *Bam*HI; E, *Eco*RI; and H, *Hin*dIII. Black arrows indicate the extension and transcription directions of the *gspC* gene and the *gspD-F* and *gspG-N* operons. Arrows below transcript arrows represent the direction and extent of cDNA after RT reactions. The short thick bars below the RT arrows indicate the nine PCR products from the corresponding RT reactions. The expected sizes of the PCR products are indicated in parenthesis in each PCR. (B) Agarose gel analysis (top) and Southern hybridization analysis (bottom) of the RT-PCR products of the *gspD-F* and *gspG-N* operons. Southern hybridization was performed using pPW2 as a probe DNA. The first lane used chromosomal DNA, the second lane used RNA, and the third lane used cDNA as a template in each PCR.

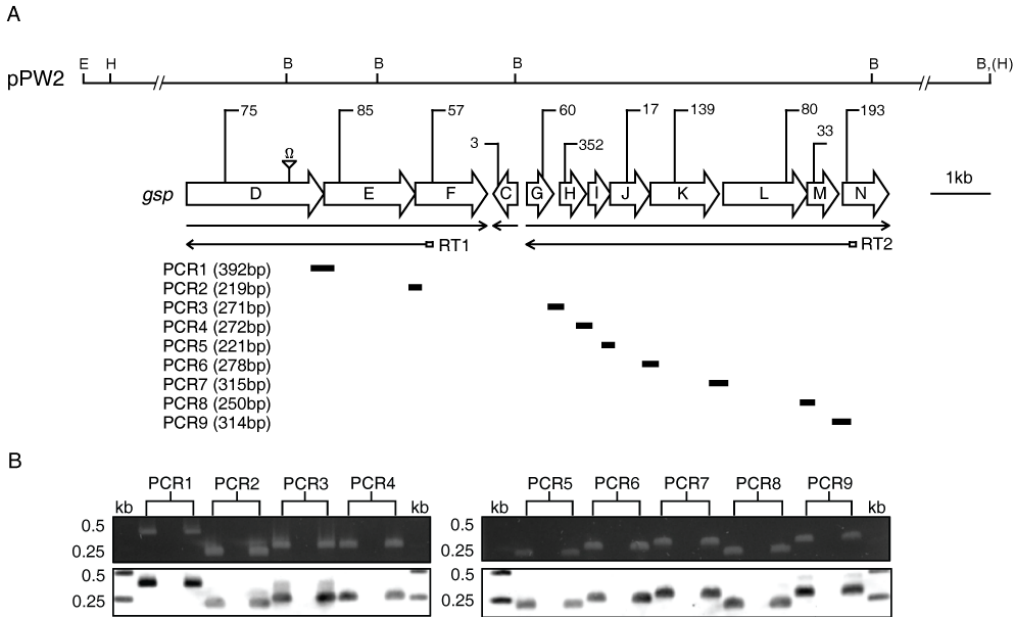

A
pPW2
E H
B
B
B
B
B,(H)
75
85
57
3
60
352
17
139
80
33
193
Ω
gsp
D
E
F
C
G
H
I
J
K
L
M
N
1kb
RT1
RT2
PCR1 (392bp)
PCR2 (219bp)
PCR3 (271bp)
PCR4 (272bp)
PCR5 (221bp)
PCR6 (278bp)
PCR7 (315bp)
PCR8 (250bp)
PCR9 (314bp)
B
PCR1
PCR2
PCR3
PCR4
PCR5
PCR6
PCR7
PCR8
PCR9
kb
0.5
0.25

Figure 6. EMSAs using purified QsmR-His and a DNA fragment containing the *gspD* promoter region (A) or the intergenic region between *gspC* and *gspG* (B). For each EMSA, 0.1 μM QsmR-His, 0.75 nM labeled target promoter DNA, 7.5 nM unlabeled target promoter DNA, and 0.75 nM unlabeled *katE* promoter DNA were used.

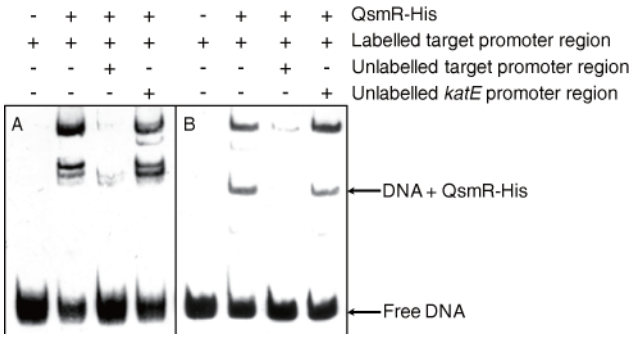
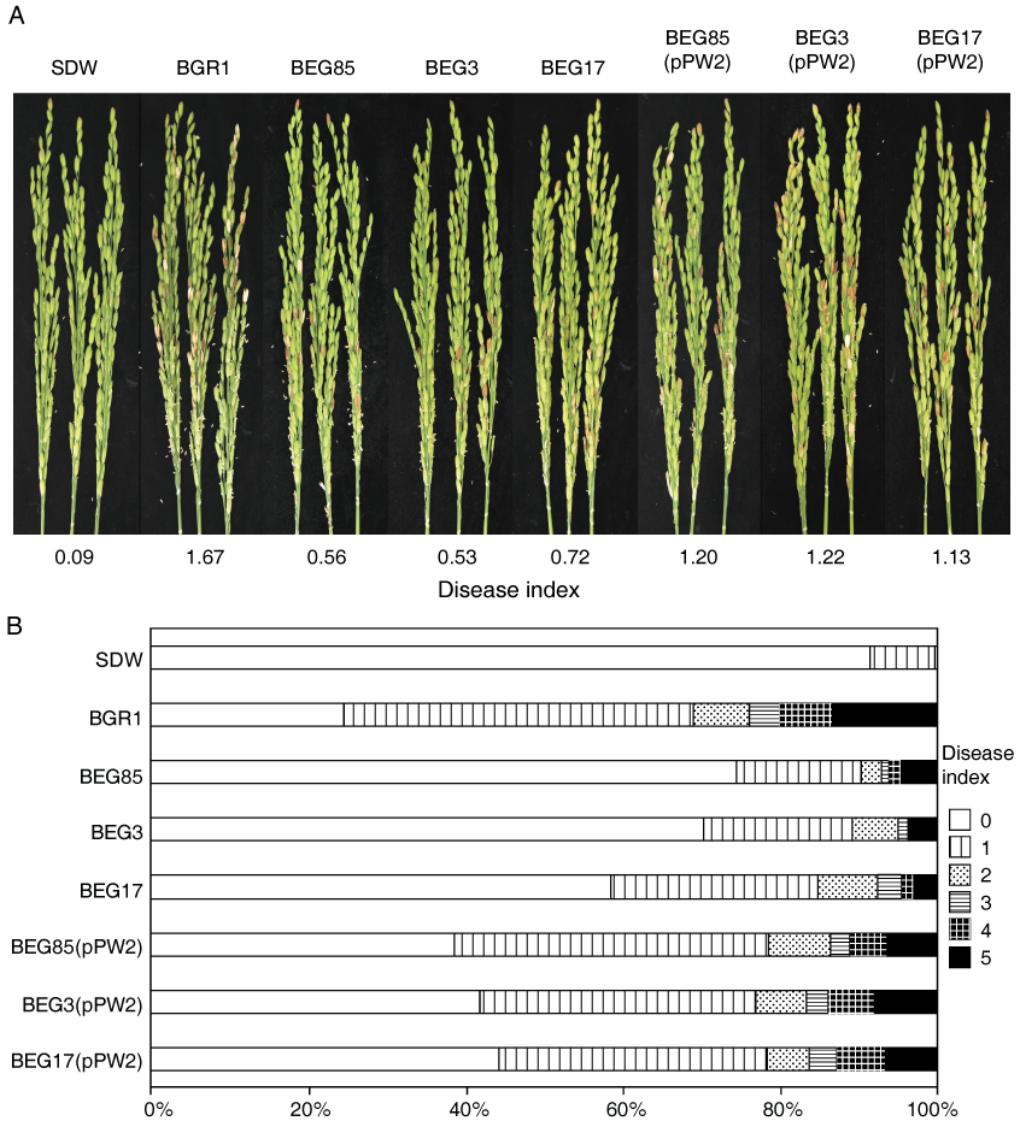

- + + + - + + + QsmR-His
+ + + + + + + + Labelled target promoter region
- - + - - - + - Unlabelled target promoter region
- - - + - - - + Unlabelled katE promoter region
A
B
DNA + QsmR-His
Free DNA

Figure 7. T2SS-deficient mutant virulence assays. Panicles of rice plants (*Oryza sativa* cv. Milyang 23) were inoculated with the wild-type BGR1, BEG85 (BGR1 *gspE*::Tn*3-gusA85*), BEG3 (BGR1 *gspC*::Tn*3-gusA3*), BEG17 (BGR1 *gspJ*::Tn*3-gusA17*), BEG85(pPW2), BEG3(pPW2), or BEG17(pPW2). Wild-type produced severe symptoms, resulting in empty heads of grain. The panicles inoculated with T2SS-deficient mutants showed significantly reduced symptoms. The photographs were taken 7 days after inoculation. The disease index of the tested rice plants is described in Materials and Methods.


A
SDW
BGR1
BEG85
BEG3
BEG17
BEG85 (pPW2)
BEG3 (pPW2)
BEG17 (pPW2)
0.09
1.67
0.56
0.53
0.72
1.20
1.22
1.13
Disease index
B
SDW
BGR1
BEG85
BEG3
BEG17
BEG85(pPW2)
BEG3(pPW2)
BEG17(pPW2)
0%
20%
40%
60%
80%
100%
Disease index
0
1
2
3
4
5

CHAPTER 2

Quorum Sensing Coordinates the Anticipation of Stationary-Phase Stress in *Burkholderia glumae*.

ABSTRACT

Many *Proteobacteria* have quorum sensing (QS) systems that control gene expression in a cell density-dependent manner. The QS systems are often mediated by *N*-acyl-homoserine lactone (AHL) to regulate diverse activities. It is common that QS-controlled genes code for production of secreted or excreted public goods. The AHLs are synthesized by members of the LuxI signal synthase family and are detected by cognate members of the LuxR family of transcriptional regulators. QS affords a means of population density-dependent gene regulation. Control of public goods via QS provides a fitness benefit. Another potential role for QS is to anticipate overcrowding. As population density increases and stationary phase approaches, QS might induce functions important for existence in stationary phase. Here we provide evidence that in two related species of the genus *Burkholderia* QS allows individuals to anticipate and survive stationary phase stress, base toxicity. Survival requires QS-dependent activation of cellular enzymes required for production of excreted oxalate, which serves to counteract ammonia-mediated alkaline toxicity during stationary phase. Our findings provide an example where QS can serve as a means to anticipate stationary phase or life at the carrying capacity of the population by activating expression of cytoplasmic enzymes,

altering cellular metabolism and producing a shared resource or public good, oxalate.

Contents of this chapter have been published in Proceedings of the National Academy of Sciences (Goo *et al*. 2012. Bacterial quorum sensing, cooperativity, and anticipation of stationary-phase stress. *Proc Natl Acad Sci* : -).

INTRODUCTION

Quorum sensing (QS) is a population density-dependent regulatory mechanism that controls a wide range of phenotypes including bioluminescence, biofilm formation, motility and virulence factor formation in many *Proteobacteria* (Fuqua *et al*., 2002; Parsek *et al*., 2005; Ng *et al*., 2009). *N*-acyl-homoserine lactone (AHL) signal molecules are synthesised most typically by members of LuxI family signal synthases. AHL concentration increases with bacterial population growth until, at high cell density, a threshold level of signal is reached. This is detected by AHL binding to receptor proteins, LuxR family of transcriptional regulators, resulting in altered gene expression (Fuqua *et al*., 2002; Parsek et al., 2005; Ng et al., 2009). A large body of work has characterized the molecular mechanisms of bacterial QS; however, it has been hard to demonstrate the population-wide benefits that drive QS-mediated cooperative behavior. Cooperative activities benefit individuals within a group (Brown *et al*., 2001; Diggle *et al*., 2007).

QS-controlled genes commonly code for production of extracellular public goods that can be shared by all members of the group regardless of which members produce them. For examples, the QS-dependent extracellular signals

in *V. fischeri* were used to control nutrient acquisition strategies in different environment (Studer *et al*., 2008). In mixed microbial habitat, QS control of antibiotic production is important for interspecies competition for limited resources (Chandler *et al*., 2012). The extracellular products from *Pseudomonas aeruginosa* have significant effects on virulence, and 3OC12-HSL produced by *P. aeruginosa* activates multiple cell types and thus potentially change the host response during infections (Smith *et al*., 2003). In *Pseudomonas aeruginosa*, fitness benefits provided by QS are relatively greater at higher cell densities because the QS-dependent extracellular public goods, proteases are produced only when they can be used efficiently (Darch *et al*., 2012). Additional potential roles of QS in bacteria have been proposed and include the hypothesis that QS enables bacteria to anticipate population carrying capacity in a given environment. Anticipation of stationary phase might allow individuals to modify their physiology in preparation for survival at population carrying capacity.

Here we address the question of whether QS is involved in anticipation of stationary-phase stress in two closely related bacteria: the rice pathogen *Burkholderia glumae*, and the non-pathogenic saprophyte *B. thailandensis*. Each species contains a conserved *N*-octanoyl homoserine lactone (C8-HSL) signaling system. In *B. glumae*, TofI-R regulates toxoflavin biosynthesis and

transport, and QsmR, an IclR-type transcriptional regulator (Kim *et al*., 2004). QsmR activates flagellar biosynthetic genes, a major catalase gene (*katG*), type II secretion system genes (*gsp*) and universal stress proteins genes (*usp*) (Kim *et al*., 2007; Chun *et al*., 2009; Goo *et al*., 2010; Kim *et al*., 2012). Less is known about gene control by C8-HSL in *B. thailandensis* via BtaI-R (Chandler *et al*., 2009). In the present study, we found QS induces cellular enzymes for production of oxalate (HOOC-COOH), which is excreted into the culture medium and is likely shared by other members of the population and as such is a public good. The oxalate protects stationary-phase cells from self-intoxication and killing as a result of ammonia production.

MATERIALS AND METHODS

I. Bacterial strains and culture conditions.

The bacterial strains and plasmids used are listed in Table 1. Strains of *B. glumae* and *B. thailandensis* were grown in LB broth (0.1% tryptone, 0.5% yeast extract, and 0.5% NaCl; USB Corp.) or in LB broth buffered with 100 mM HEPES (pH 7.0) at 37°C. C8-HSL was supplemented to a final concentration of 1 μM at the beginning of each growth experiment, where indicated. Antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 20 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; nalidixic acid, 20 μg mL^{-1}; rifampicin, 50 μg mL^{-1}; spectinomycin, 25 μg mL^{-1}; and tetracycline, 10 μg mL^{-1}.

II. Nucleic acid manipulations.

Standard methods were used for DNA cloning, restriction mapping, and gel electrophoresis (Sambrook *et al*., 1989). Vector DNA was treated with the appropriate restriction enzymes as recommended by the suppliers (New England Biolabs, Ipswich, MA, USA), and extraction of the DNA fragments

from the gels was carried out as described by the manufacturer (Qiagen, Valencia, CA, USA). A previously constructed cosmid genomic library was used (Kim *et al*., 2004). The pLAFR3 and pLAFR6 derivatives were mobilized into the *B. glumae* strains by triparental mating (Figurski *et al*., 1979).

III. RNA extraction and sequencing.

Strains of *B. glumae* BGR1, BGS2 (BGR1 *tofI*::Ω), and BGS9 (BGR1 *qsmR*::Ω) were grown in LB broth (Difco) at 37°C overnight and diluted to approximately 1×10^7 cells per ml. Total RNA was extracted after cells were grown for an additional 6 or 10 h using the RNeasy midi kit (Qiagen) following the manufacturer's protocol. Total RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA) and ribosomal RNA was removed by using a MICROBExpress™ Kit (Ambion, Carlsbad, CA, USA). The quantity and quality of the RNA were evaluated after the rRNA removal by using RNA electropherograms (Agilent 2100 Bioanalyzer) and the RNA integrity number (RIN) (Schroeder *et al*., 2006). We used 100 ng enriched mRNA in the mRNA-Seq sample preparation kit (Illumina) for library construction following the manufacturer's protocols. Deep sequencing was performed by using two runs of the Illumina Genome Analyzer IIx to generate non-directional, single-ended 36-bp reads. Quality-filtered reads were mapped to the reference

genome sequences using CLC Genomics Workbench 4.0 (CLC bio). The relative transcript abundance was measured in reads per kb of exon per million mapped sequence reads (RPKM) (Mortazavi *et al*., 2008).

IV. Transposon mutagenesis and Southern hybridization

The pOBC1 carrying all *obcAB* (oxalate biosynthesis component) genes from *B. glumae* BGR1 were mutagenized with Tn*3-gusA* as described by Bonas *et al.* (1989). The insertion site and orientation of the Tn*3-gusA* in each mutant were determined by restriction digests and direct sequencing of the plasmid using the primer Tn3gus (5'-CCGGTCATCTGAGACCATTAAA AGA-3'). The mutagenized plasmids carrying Tn*3-gusA* insertions were introduced individually into the parent strain by conjugation followed by marker-exchange into the chromosome as described by Fellay *et al.* (1989). All marker-exchanges were confirmed by Southern hybridization analysis. For Southern hybridization, total genomic DNA was digested with appropriate restriction enzymes (New England Biolabs, Ipswich, MA, USA), separated on a 0.7% w/v agarose gel by electrophoresis, and transferred to Hybond^{TM} N+ nylon membranes (GE Healthcare, Uppsala, Sweden) using a Trans-Blot SD semi-dry electrophoretic transfer cell according to the manufacturer's recommendations (Bio-Rad, Hercules, CA, USA). The southern hybridizations

were performed as described previously (Sambrook *et al*., 1989).

V. β-Glucuronidase assays.

The β-glucuronidase enzyme assay was performed as described previously, with some modifications (Jefferson *et al*., 1987). All *B. glumae* derivatives were grown in LB medium, centrifuged, resuspended in GUS extraction buffer, and lysed using sonication with a VCX-400 sonicator (Sonics & Materials Inc., CT, USA). The extract was used in the *β*-glucuronidase enzyme assay with 4-methylumbelliferyl glucuronide as the substrate. The fluorescence was measured at 365 nm excitation and 460 nm emission in a TKO100 fluorometer (Hoefer Scientific Instruments, San Francisco, USA). One unit of β-glucuronidase was defined as 1 nm of released 4-methylumbelliferon per bacterium per minute.

VI. Ammonia and Oxalate Measurements.

We used an Ion-Selective Electrode Meter and gas-sensing ammonia electrode (Thermo Scientific, Waltham, MA, USA) to detect the gas phase of ammonia after addition of the alkaline reagent to the culture medium. To

measure oxalate, we used a diagnostic kit (Libios, Bully, France) as described by the manufacturer (Laker *et al*., 1980). In short, oxalate was converted to carbon dioxide and hydrogen peroxide by oxalate oxidase and hydrogen peroxide was measured by reaction with 3-(dimethylamino) benzoic acid to form a blue colored compound, catalyzed by peroxidase, and the absorbance at 590 nm was measured.

VII. Electrophoretic mobility shift assays.

QsmR-His was purified as described previously (Kim *et al*., 2007). The 216-bp upstream region of *obcAB* was PCR-amplified using pobc-F (5′-GAACGGCCCTCTCTCTATGG-3′) and pobcR1 (5′-ACATTCGGCGACT TATTTCCC-3′) primers. The PCR product was labeled with biotin by using a Lightshift Chemiluminescent Electrophoretic Mobility Shift Assay Kit, as described by the manufacturer (Pierce, Rockford, IL, USA). For non-specific competitor DNA, the 242-bp upstream region of *katE* was amplified as described previously (Kim *et al*., 2007). Purified QsmR-His (200 nM) was incubated with 3 nM of biotin-labeled DNA in binding buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1 mM EDTA] for 15 min at 28°C. Unlabeled target DNA (30 nM) and a non-specific unlabeled competitor DNA (3 nM) were added to the binding reaction. The reaction mixtures were separated on a

non-denaturing 4% polyacrylamide gel and transferred to nitrocellulose membranes followed by detection with streptavidin horseradish peroxidase/chemiluminescence, as described by the manufacturer (Pierce).

RESULTS

I. QS is Essential for Stationary Phase Survival of *Burkholderia*.

We assessed the role of QS during stationary phase in nutrient-rich Luria-Bertani (LB) broth by using wild-type and C8-HSL-QS signal synthesis mutants of *B. glumae* and *B. thailandensis*. Exponential growth of each QS mutant was comparable to its isogenic wild-type strain; however, whereas the wild-type strain survived long periods in stationary phase, the QS mutants showed massive and rapid population crashes commencing shortly after onset of stationary phase (Fig. 1A). Mutant population crashes were averted by addition of C8-HSL to the growth media (Fig. 1A). This indicated that QS is involved in stationary-phase survival in these two bacterial species.

II. Massive Population Crashes are Due to Medium Alkalization in Stationary Phase.

To determine the cause of the observed population crashes, we monitored the pH of the culture fluids during growth. Early in growth, the pH of both the wild-type and QS mutants of *B. glumae* and *B. thailandensis* was

about 7 and then rose to between 7.5 and 8 as cultures entered stationary phase (Fig. 1B). During continued stationary-phase growth, the pH of wild-type cultures fell to 7 or below, whereas the pH of the QS mutant cultures rose above 8. The pH increase of the QS mutants correlated with a drop in cell viability (Fig. 1).

To test whether cell death was caused by base toxicity, we added 100 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES] at pH 7 to the growth medium. We found that addition of this strong buffer to the growth medium spared the QS mutants from death in stationary phase (Fig. 2). We also adjusted the culture medium pH with sodium hydroxide and monitored cell growth to determine sensitivity of *B. glumae* and *B. thailandensis* in alkaline conditions. We found that the wild-type bacteria survived at pH 8 but not at pH 9 (Fig. 3). These data support the view that culture medium alkalization is the cause of QS mutant cell death.

It is well documented in other bacteria that alkalization occurs due to ammonia production as a result of amino acid catabolism in complex media like LB broth (Sezonov *et al*., 2007; McFall *et al*., 1996). Thus, we monitored ammonia production in the wild type and C8-HSL-QS signal synthesis mutants of *B. glumae* and *B. thailandensis* grown in LB broth. We found that ammonia was produced continuously by both wild type and QS mutants (Fig. 4A).

However, ammonia production of QS mutants seemed to decrease 12 h after incubation in LB but not in buffered LB (Figs. 4A and 5A). The decrease in ammonia production corresponds with the growth defect of the mutants (Fig. 1A). Thus, the QS-dependent shift in metabolism that limits alkalization is not a shift away from ammonia production. Instead, we posit that QS controls metabolism such that pH changes are constrained.

III. QS-Dependent Oxalate Production Counteracts Base Toxicity.

To identify the QS-controlled process or processes involved in countering ammonia-induced alkalization, we performed an RNAseq transcriptome comparison of wild-type *B. glumae* and its QS mutant strain. We also used published *B. glumae* proteomics data (Goo *et al*., 2010). The results showed that among the QS-activated genes were those encoding the enzymes ObcA and ObcB (Table 2). *obcA* and *obcB* comprise a two-gene operon and are responsible for oxalate synthesis (Nakata *et al*., 2010, Figs. 6A and B). Oxalate is an acidic molecule found in multiple domains of life-its physiological role in bacteria has been largely uncharacterized and is predicted to exist primarily as a metabolic end product. We hypothesized that the acidity of oxalate might counter base intoxication in these species. Thus, we measured oxalate levels in wild type and QS mutant cultures of *B. glumae* and *B. thailandensis*. The wild

types of each species produced oxalate in amounts presumably sufficient to neutralize the accumulated ammonia. The QS mutants produced very little oxalate (Fig. 4B). This indicates that QS is required for production of a neutralizing agent, oxalate, in wild-type bacteria to avoid alkaline toxicity. However, alkaline conditions were not required for oxalate synthesis (Fig. 5B).

Because ObcA and ObcB are required for production of oxalate in *B. glumae* (Nakata *et al*., 2010; Li *et al*., 1999), we hypothesized that *obcA* and *obcB* mutants would also be subject to ammonia-induced base toxicity in stationary phase. As expected, both *obcA* and *obcB* mutants showed massive population crashes corresponding with increased pH in stationary phase (Fig. 6C). In *B. thailandensis*, a single Obc homolog, Obc1, is predicted to be responsible for oxalate biosynthesis (Nakata *et al*., 2011). The N-terminus of Obc1 shares 54% amino acid identity with full-length ObcA, whereas the C-terminus of Obc1 has no similarity with ObcA or ObcB (Fig. 7). We measured oxalate levels in the wild type and an *obc1* mutant of *B. thailandensis*. We found that the *B. thailandensis obc1* mutant did not produce oxalate, confirming that Obc1 is responsible for oxalate biosynthesis in *B. thailandensis* (Fig. 6D). Consistent with the hypothesis that oxalate is required for stationary phase survival, the *obc1* mutant of *B. thailandensis* exhibited alkalization and a massive population crash in stationary phase (Fig. 6E).

IV. Regulation of *obc* Genes by QsmR.

Because our results show that oxalate production in *B. glumae* and *B. thailandensis* requires a functional C8-HSL QS system, we asked whether the *obc* operon was directly or indirectly activated by QS. Insight came from our transcriptomics analysis of *B. glumae*, which showed that a functional C8-HSL QS system and also the QsmR regulator were required for activation of *obcA* and *obcB* (Table 2). These data indicate that the *obc* genes belong to the QsmR regulon, which is activated by QS. To further confirm this hypothesis, we measured expression levels of *obcA* and *obcB* from a chromosomal *obcAB-gusA* transcriptional fusion in *B. glumae*. We found that QsmR activates *obcA* and *obcB* expression and interacted directly with the *obcA-B* promoter (Fig. 8).

V. *B. glumae* and *B. thailandensis* QsmR Mutants Behave like QS Mutants.

Because the *B. glumae qsmR* and QS mutants show similar low levels of *obcAB* expression, we reasoned that *qsmR* mutants might fail to accumulate oxalate and experience stationary phase death as do QS mutants. Thus, we tested cell viability of *B. glumae* and *B. thailandensis qsmR* mutants grown in

LB broth, and showed the mutants have a stationary phase survival defect (Fig. 1). The *qsmR* mutant population crashes correlate with rising pH and a lack of oxalate production (Figs. 1 and 4). Therefore, we believe C8-HSL QS controls a battery of genes, including *qsmR* and it is the *qsmR* product that activates genes for oxalate synthesis, directly.

DISCUSSION

There has been recent debate about the selective forces that led to the evolution of AHL QS. One idea is that QS control of public goods provides an advantage to cooperating individuals (Diggle *et al*., 2007; Williams *et al*., 2007; Keller *et al*., 2006; West *et al*., 2006). We believe we have uncovered another particularly interesting role for AHL QS in *B. glumae* and *B. thailandensis*. The enormous population crashes of QS mutants in stationary phase represent a case where QS serves to anticipate crowding before the cells experience loss of viability. These findings suggest that bacteria use QS to determine the population density to anticipate carrying capacity to avoid population collapse. Specifically, the two species of *Burkholderia* we studied sense increasing population densities by QS and alter their metabolism; thus, the group is protected from high concentrations of a toxic end product of energy metabolism, ammonia. The metabolic rewiring induced by QS leads to excretion of oxalate, a public good that provides protection from base toxicity to the group.

When bacteria use amino acids as a carbon source, deamination results in ammonia release, which can result in alkalization (Sezonov *et al*., 2007; McFall *et al*., 1996). For many bacterial species ammonia accumulation is not a direct cause of population crashes in stationary phase because pH homeostasis mechanisms allow cells to survive the alkaline conditions. However, we found

that alkaline pH is toxic to wild type strains of *B. glumae* and *B. thailandensis* and that these species use C8-HSL QS to limit environmental alkalization. QS induces a change in metabolism such that cells excrete copious amounts of oxalate. In a sense, oxalate is an ideal organic acid for neutralizing ammonia because of its dianionic nature. Therefore, QS control of cellular metabolism serves an anticipatory function and involves the production of an excreted public good, oxalate.

It has been reported in some bacteria that QS controls carbon flux and this subsequently results in the maintenance of neutral pH during culture (Van Houdt *et al*., 2006; Grignon *et al*., 1991). We believe, however, that QS control of oxalate in these *Burkholderia* species represents a novel mechanism of anticipatory pH control. Notably, QS-controlled oxalate accumulation begins in late exponential phase, long before the toxic effects of ammonia-induced alkalinization lead to reduced cell viability. Additionally, oxalate is likely not an excreted product later imported and used for carbon and energy, as it cannot serve as a carbon source for *B. thailandensis* nor *B. glumae.*

The fact that oxalate is produced at neutral pH suggests that anticipatory production of oxalate by QS-dependent mechanisms is neither dependent on alkaline pH nor operated by a simple pH-dependent regulatory mechanism. Where might *B. glumae* and *B. thailandensis* encounter alkaline

pH in natural habitats? It is plausible that *B. glumae* experiences high pH in the plant apoplast due to active defense responses in plants upon infection. There are sugars and organic acids rather than amino acids in apoplasts (Nachin *et al*., 2002). But under apoplastic conditions, plants alkalize the infection site as a defense mechanism (Byers *et al*., 2002). In this regard, it is conceivable that QS-dependent oxalate production would contribute to the fitness of *B. glumae* by avoiding plant-mediated alkalization in plant apoplast during infection. Furthermore, the production of oxalate has been linked to pathogenic *B. glumae* isolates. A survey of 200 isolates from Japan showed that all 180 virulent species produced oxalate whereas the 20 avirulent isolates did not (Li *et al*., 1999). In addition, avoidance of alkalization would be important because AHLs are degraded above pH 8.0 (Yates *et al*., 2002; Coe *et al*., 2005). Therefore, anticipatory production of oxalate in a QS-dependent manner might be one way of protecting AHL signals in alkaline conditions. These two species exist in soil where pH can be variable.

Much of the literature on biological oxalate synthesis has focused on formation of oxalate crystals in the human body that cause kidney stones, or crystal formation in plants (Baker *et al*., 2004; Green *et al*., 2005; Williams *et al*., 2011). Oxalate production by certain plant pathogenic fungi is important for virulence (Chen *et al*., 2010; Rogul *et al*., 1972). Oxalate has traditionally

been considered as a metabolic end product, but it is clear that, in the *Burkholderia* species we studied, oxalate serves to protect against base toxicity. This is consistent with a previous report that growth inhibition of *B. pseudomallei* is due to ammonium toxicity following spontaneous loss of oxalate production (Kasai *et al*., 1963).

In *B. glumae*, both ObcA and ObcB synthesize oxalate from acetyl-CoA and oxaloacetate derived from the tricarboxylic acid (TCA) cycle (Nakata *et al*., 2010; Li *et al*., 1999). Oxalate synthesis in bacteria more commonly occurs by a separate pathway involving glyoxylate as an intermediate (Singh *et al*., 2009; Laker *et al*., 1980), however, we did not find evidence for this pathway in any of the two *Burkholderia* species. We show that Obc1 is responsible for oxalate synthesis in *B. thailandensis*. In support of the conserved functionality among the Obc enzymes, the *B. mallei obc1* gene can complement a *B. glumae obcA* mutation (Nakata *et al*., 2011). Therefore, oxalate synthesis in *B. glumae* and *B. thailandensis* seems to share a biochemical pathway in that oxalate is made in the branched TCA cycle in a QS-dependent manner.

The results of this work show that QS can function to allow anticipation of overcrowding and promote bacterial survival at maximum population carrying capacity. It lends insight into how QS bacteria have evolved to control

both public and private goods. One can imagine that QS evolved in a common ancestor of *B. glumae* and *thailandensis* to anticipate the onset of stationary phase and prepare cells to survive in this crowded environment. Survival involves induction of cellular functions that are required for production of oxalate, an excreted public good. This is one solution to a problem encountered by bacteria that use amino acids for energy. The problem being an end product of amino acid metabolism is ammonia and ammonia production leads to increasing pH in a local environment.

LITTERATURE CITED

Baker PR, Cramer SD, Kennedy M, Assimos DG. Holmes RP (2004) Glycolate and glyoxylate metabolism in HepG2 cells. *Am J Physiol Cell Physiol* 287:C1359-1365.

Bonas U, Stall RE, Staskawicz BJ (1989) Genetic and structural characterization of the avirulence gene avrBs3 from *Xanthomonas campestris* pv. *vesicatoria*. *Mol Gen Genet* 218:127-136.

Brett PJ, DeShazer D, Woods DE (1997) Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. *Epidemiol Infect* 118:137-148.

Brown SP, Johnstone RA (2001) Cooperation in the dark: signaling and collective action in quorum-sensing bacteria. *Proc Biol Sci* 268:961–965.

Byers JT, Luca C, Salmond GPC, Welch M (2002) Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signal molecule. *J Bacteriol* 184:1163-1171.

Chandler JR, et al. (2009) Mutational analysis of *Burkholderia thailandensis* quorum sensing and self-aggregation. *J Bacteriol* 191:5901-5909.

Chandler JR, Heilmann S, Mittler JE, Greenberg EP (2012) Acyl-homoserine lactone-dependent eavesdropping promotes competition in a

laboratory co-culture model. *ISME J* doi: 10.1038/ismej.2012.69.

Chen C, Sun Q, Narayanan B, Nuss DL, Herzberg O (2010) Structure of oxalacetate acetylhydrolase, a virulence factor of the chestnut blight fungus. *J Biol Chem* 285:26685-26696.

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

Coe FL, Evan A, Worcester E (2005) Kidney stone disease. *J Clin Invest* 115:2598–2608.

Darch SE, West SA, Winzer K, Diggle SP (2012) Density-dependent fitness benefits in quorum sensing bacterial populations. *Proc Natl Acad Sci USA* 109:8259-8263.

Diggle SP, Gardner A, West SA, Griffin AS (2007) Evolutionary theory of bacterial quorum sensing: When is a signal not a signal? *Philos Trans R Soc Lond B Biol Sci* 362:1241–1249.

Diggle SP, Griffin AS, Campbell GS, West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450:411–414.

Fellay R, Krisch HM, Prentki P, Frey J (1989) Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning

of genes in gram-negative bacteria. *Gene* 76:215-226.

Fuqua C, Greenberg EP (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* 3:685-695.

Gallagher L, Manoil C (2012) *Burkholderia thailandensis* mutant library. Available from: www.gs.washington.edu/labs/manoil/burkholderia.htm. Accessed November 1, 2012.

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.

Green MA, Fry SC (2005) Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-O-oxalyl-L-threonate. *Nature* 433:83-87.

Grignon C, Sentenac H (1991) pH and ionic conditions in the apoplast. *Annu Rev Plant Physiol Plant Mol Biol* 42:103-128.

Kasai T, Suzuki I, Asai T (1963) Glyoxylate oxidation in *Acetobacter* with reference to the formation of oxalic acid. *J Gen Appl Microbiol* 9:49-58.

Keller L, Surette MG (2006) Communication in bacteria: An ecological and evolutionary perspective. *Nat Rev Microbiol* 4:249–258.

Kim H, Goo E, Kang Y, Kim J, Hwang I (2012) Regulation of universal stress protein genes by quorum sensing and RpoS in *Burkholderia glumae*. *J Bacteriol*. 914:982-992.

Kim J, et al. (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, et al. (2007) Regulation of polar flagellum genes is mediated by quorum sensing and FlhDC in *Burkholderia glumae*. *Mol Microbiol* 64:165-179.

Laker MF, Hofmann AF, Meeuse BJ (1980) Spectrophotometric determination of urinary oxalate with oxalate oxidase prepared from moss. *Clin Chem* 26:827-830.

Li HQ, Matsuda I, Fujise Y, Ichiyama A (1999) Short-chain acyl-CoA-dependent production of oxalate from oxaloacetate by *Burkholderia glumae*, a plant pathogen which causes grain rot and seedling rot of rice via the oxalate production. *J Biochem* 126:243-253.

McFall E, Newman EB (1996) Amino acids as carbon sources, p358-379. In: Neidhardt FC, Curtis R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaschter M, Umbarger HE (eds) *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd edn. ASM, Washington, DC.

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nat*

Methods 5:621-628.

Nachin L, Barras F (2000) External pH: An environmental signal that helps to rationalize *pel* gene duplication in *Erwinia chrysanthemi*. *Mol Plant Microbe Interact* 13:882-886.

Nakata PA (2011) The oxalic acid biosynthetic activity of *Burkholderia mallei* is encoded by a single locus. *Microbiol Res* 166:531-538.

Nakata PA, He C (2010) Oxalic acid biosynthesis is encoded by an operon in *Burkholderia glumae*. *FEMS Microbiol Lett* 304:177-182.

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

Parsek MR, Greenberg EP (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiol* 13:27-33.

Rogul M, Carr SR (1972) Variable ammonia production among smooth and rough strains of *Pseudomonas pseudomallei*: resemblance to bacteriocin production. *J Bacteriol* 112:327-380.

Schroeder A, et al. (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* 7:3.

Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in Luria-Bertani broth. *J Bacteriol* 189:8746-8749.

Singh R, et al. (2009) An ATP and oxalate generating variant

tricarboxylic acid cycle counters aluminum toxicity in *Pseudomonas fluorescens*. *PLoS One* 4:e7344.

Smith RS, Iglewski BH (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* 6:56-60.

Staskawicz B, Dahlbeck D, Keen N, Napoli C (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* 169:5789-5794.

Studer SV, Mandel MJ, Ruby EG (2008) AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. *J Bacteriol* 190:5915-5923.

Ulrich RL, et al. (2004) Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *J Med Microbiol* 53:1053-1064.

Ulrich RL, Hines HB, Parthasarathy N, Jeddeloh JA (2004) Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. *J Bacteriol* 186:4350-4360.

Van Houdt R, Aertsen A, Michiels C (2007) Quorum-sensing-dependent switch to butanediol fermentation prevents lethal medium acidification in *Aeromonas hydrophila* AH-1N. *Res Microbiol* 158:379-385.

West SA, Griffin AS, Gardner A, Diggle SP (2006) Social evolution theory for microorganisms. *Nat Rev Microbiol* 4:597–607.

Williams B, Kabbage M, Kim HJ, Britt R, Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog* 7:e1002107.

Williams P, Winzer K, Chan WC, Cámara M (2007) Look who's talking: Communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* 362:1119–1134.

Yates EA, et al. (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* 70:5635-5646.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Characteristics[a]	Source or Reference
B. glumae		
BGR1	Wild type, Rif^R	Kim *et al*., 2004
BGS2	BGR1 *tofI*::Ω	Kim *et al*., 2004
BGS9	BGR1 *qsmR*::Ω	Kim *et al*., 2007
BOBA83	BGR1 *obcA*::Tn*3-gusA83*	This study
BOBB29	BGR1 *obcB*::Tn*3-gusA29*	This study
B. thailandensis		
E264	Wild type	Brett *et al*., 1997
JBT101	E264 Δ*btaI1*	Chandler *et al*., 2009
BT09539	E264 *qsmR*-135::ISlacZ-PrhaBo-Tp/FRT	Gallagher *et al*., 2012
BT00401	E264 *obc1*-117::ISlacZ hah-Tc	Gallagher *et al*., 2012
pLAFR3	Tra^-, Mob^+, RK2 replicon, Tet^R	Staskawicz *et al*., 1987
pOBC1	23.2 kb DNA fragment from strain BGR1 cloned into pLAFR3	This study

[a] Rif^R, rifampicin resistance; Tet^R, tetracycline resistance.

Table 2. Normalized RNAseq results of *B. glumae obcAB* genes.

Gene	Locus_ID	Mid-exponential growth stage (6 h after subculture)			Late-exponential growth stage (10 h after subculture)		
		BGR1 (WT)	BGS2 (*tofI*::Ω)	BGS9 (*qsmR*::Ω)	BGR1 (WT)	BGS2 (*tofI*::Ω)	BGS9 (*qsmR*::Ω)
obcA	bglu_2g18790	6665	118	1159	36111	9387	1468
obcB	bglu_2g18780	4338	113	1033	29740	7202	1413

Figure 1. Cell viability and culture medium pH of *B. glumae and B. thailandensis* grown in LB broth at 37°C with shaking. At the indicated times, small volumes were removed and used to determine cell numbers as colony-forming units by plate counting methods (*A*) or cells were removed by centrifugation and pH measured with a pH electrode and meter (*B*). Open blue circles, wild-type strains of two *Burkholderia* sp.; open orange triangles, C8-HSL synthase mutants: BGS2 (*B. glumae* BGR1 *tofI*::Ω) and JBT101 (*B. thailandensis* E264 Δ*btaI1*); open green squares, QsmR mutants: BGS9 (*B. glumae* BGR1 *qsmR*::Ω) and BT09539 (*B. thailandensis* E264 *qsmR*-135::ISlacZ-PrhaBo-Tp/FRT); filled red triangles, C8-HSL synthase mutants grown in media containing 1 μM C8-HSL. Error bars represent the error ranges of triplicate experiments.

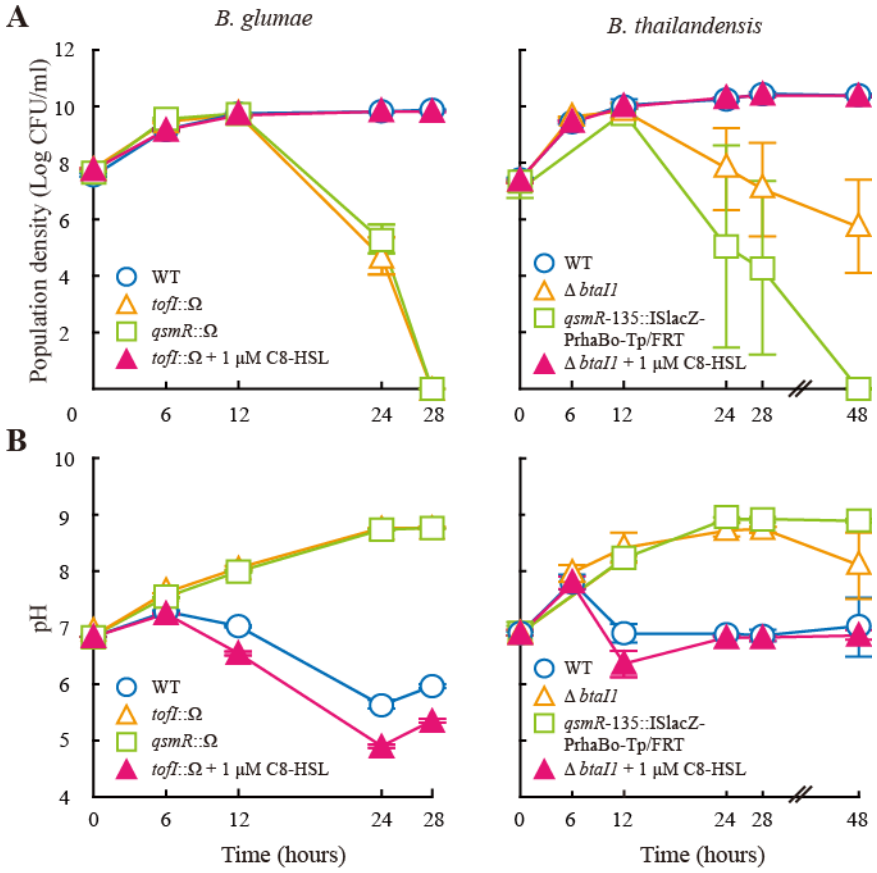

A
B. glumae
B. thailandensis
Population density (Log CFU/ml)
WT
tofI::Ω
qsmR::Ω
tofI::Ω + 1 μM C8-HSL
WT
Δ btaI1
qsmR-135::ISlacZ-PrhaBo-Tp/FRT
Δ btaI1 + 1 μM C8-HSL
B
pH
Time (hours)
Time (hours)

Figure 2. Viability and culture medium pH of *B. glumae* and *B. thailandensis* grown in LB supplemented with 100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] at pH 7.

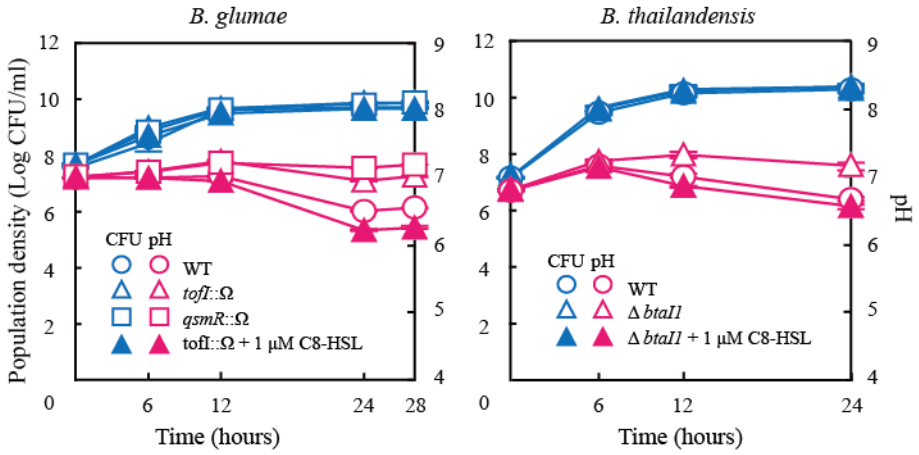

B. glumae
B. thailandensis
Population density (Log CFU/ml)
pH
Time (hours)
Time (hours)
CFU pH
WT
tofI::Ω
qsmR::Ω
tofI::Ω + 1 μM C8-HSL
CFU pH
WT
Δ btaI1
Δ btaI1 + 1 μM C8-HSL

Figure 3. Viability of wild type *B. glumae* and *B. thailandensis* in LB broth at different starting culture pH values.

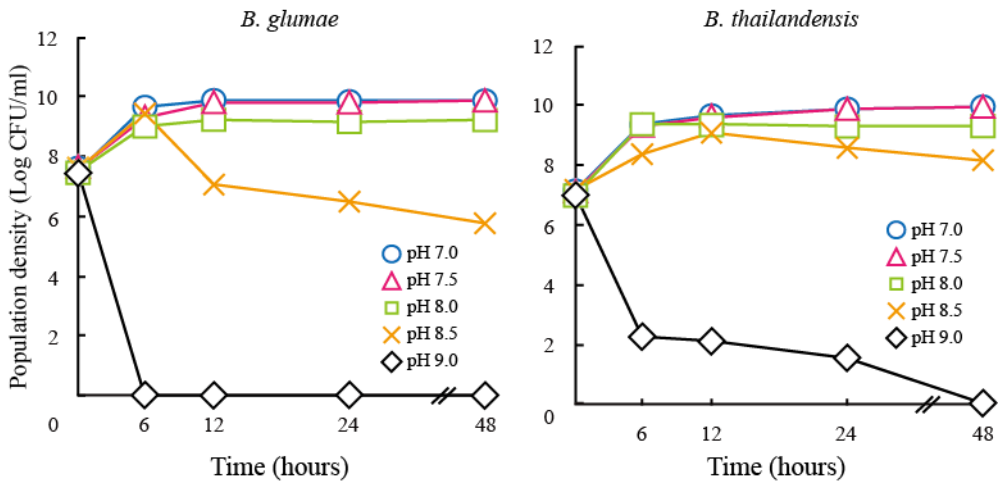

B. glumae
B. thailandensis
Population density (Log CFU/ml)
Time (hours)
Time (hours)
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
0
2
4
6
8
10
12
6
12
24
48

Figure 4. Ammonia and oxalate production by *B. glumae* and *B. thailandensis*. Cultures were grown as described in the Fig. 1 legend and concentrations of ammonia (A) or oxalate (B) were measured as described in the Materials and Methods. Open circles, the wild- type strains; open triangles, C8-HSL synthase mutants; open squares, QsmR mutants; filled triangles, C8-HSL synthase mutants grown in media containing 1 μM C8-HSL. Error bars represent the error ranges of triplicate experiments.

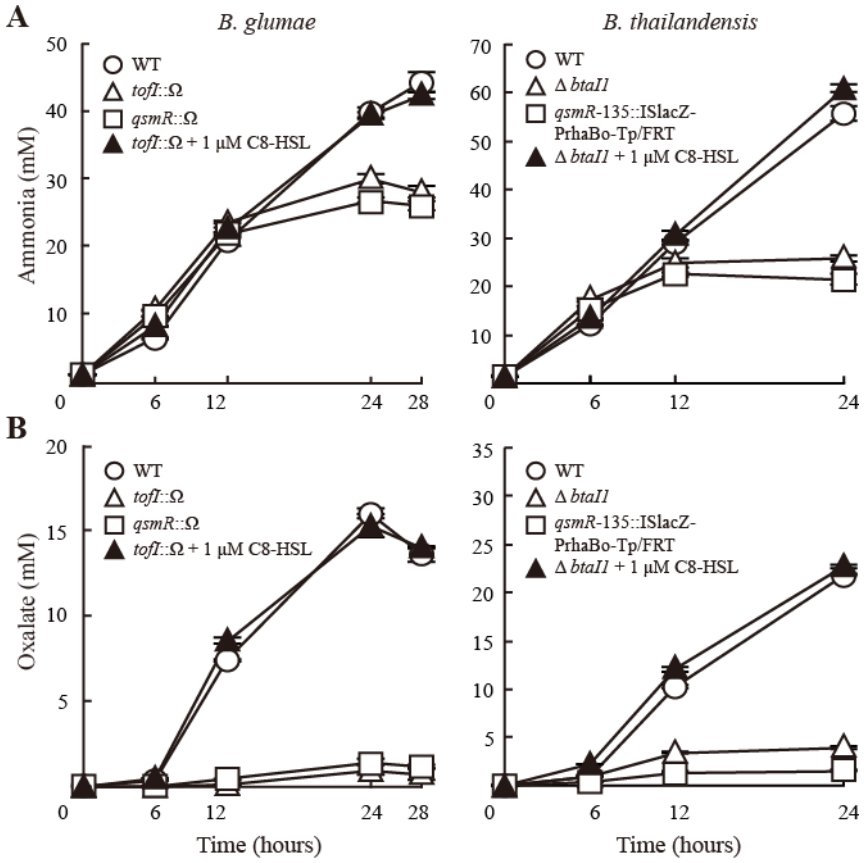

A
B. glumae
B. thailandensis
WT
tofI::Ω
qsmR::Ω
tofI::Ω + 1 μM C8-HSL
Δ btaI1
qsmR-135::ISlacZ-PrhaBo-Tp/FRT
Δ btaI1 + 1 μM C8-HSL
Ammonia (mM)
B
Oxalate (mM)
Time (hours)

Figure 5. Ammonia and oxalate levels in culture fluid of the wild type, the *tofI*, and the *qsmR* mutant of *B. glumae* grown in LB supplemented with 100 mM HEPES. Open circles, the wild- type strains; open triangles, C8-HSL synthase mutants; open squares, QsmR mutants. Error bars represent the error ranges of triplicate experiments.

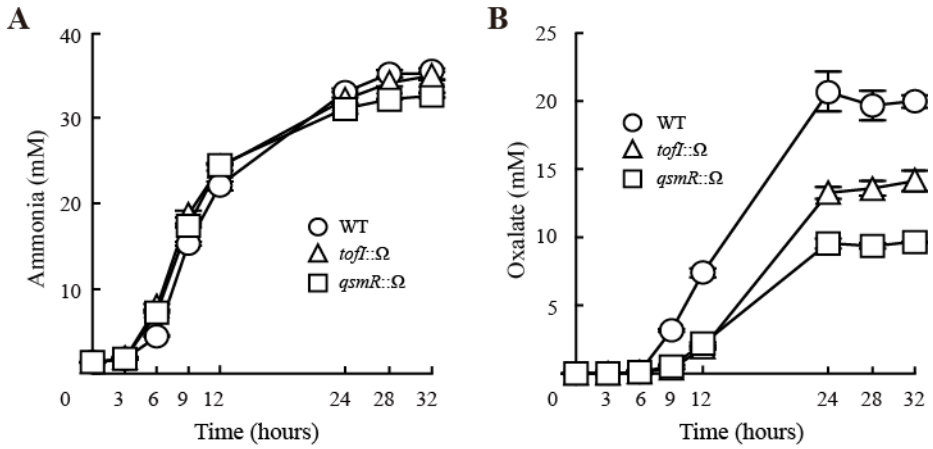

A
Ammonia (mM)
40
30
20
10
0
3
6
9
12
24
28
32
Time (hours)
WT
tofI::Ω
qsmR::Ω
B
Oxalate (mM)
25
20
15
10
5
0
3
6
9
12
24
28
32
Time (hours)
WT
tofI::Ω
qsmR::Ω

Figure 6. Massive population crashes due to alkaline toxicity in *obcAB* and *obc1 B. glumae* and *B. thailandensis* mutants, respectively. (A) Organization of *obcA* and *obcB* genes in *B. glumae*. Vertical bars indicate the positions and orientations of the Tn*3*-*gusA* insertions. Oxalate production is indicated above the restriction map. N, *Not*I; E, *Eco*RI; H, *Hin*dIII. (B and D) Oxalate concentration and (C and E) cell numbers (blue) and pH (red) in cultures grown as described in Figs. 1 and 2 legends. Strains were the wild-type *B. glumae* BGR1 and *B. thailandensis* E264 (blue circles), BOBA83 (*B. glumae* BGR1 *obcA*::Tn*3*-*gusA83*) and BT00401 (*B. thailandensis* E264 *obc1*-117::ISlacZ hah-Tc) (red triangles), and BOBB29 (*B. glumae* BGR1 *obcB*::Tn*3*-*gusA29*) (orange squares). Error bars represent the error ranges of triplicate experiments.

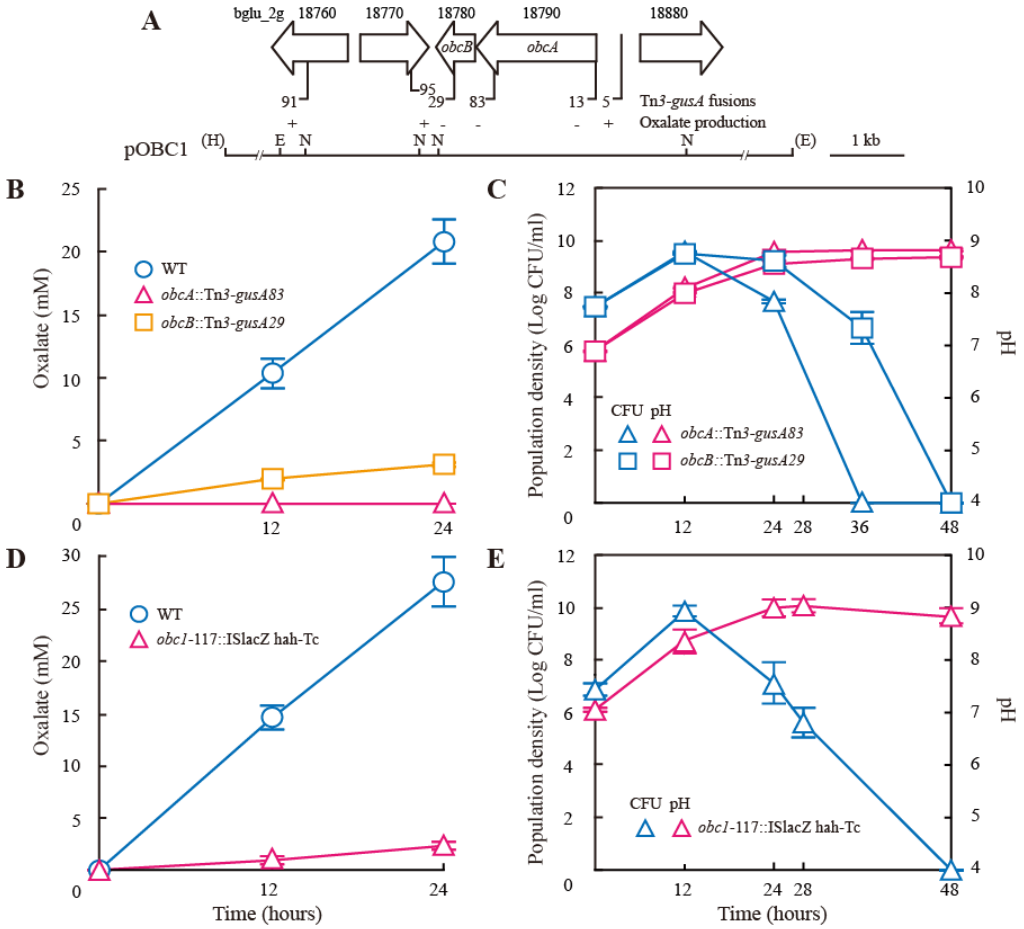

A
bglu_2g
18760
18770
18780
18790
18880
obcB
obcA
91
95
29
83
13
5
Tn3-gusA fusions
Oxalate production
pOBC1
(H)
E
N
N N
N
(E)
1 kb
B
Oxalate (mM)
WT
obcA::Tn3-gusA83
obcB::Tn3-gusA29
C
Population density (Log CFU/ml)
pH
CFU pH
obcA::Tn3-gusA83
obcB::Tn3-gusA29
D
Oxalate (mM)
WT
obc1-117::ISlacZ hah-Tc
Time (hours)
E
Population density (Log CFU/ml)
pH
CFU pH
obc1-117::ISlacZ hah-Tc
Time (hours)

Figure 7. Comparison of the oxalate biosynthetic genes in *B. glumae*, *B. pseudomallei*, and *B. thailandensis*. Numbers in parenthesis indicate amino acid numbers encoded by each gene.

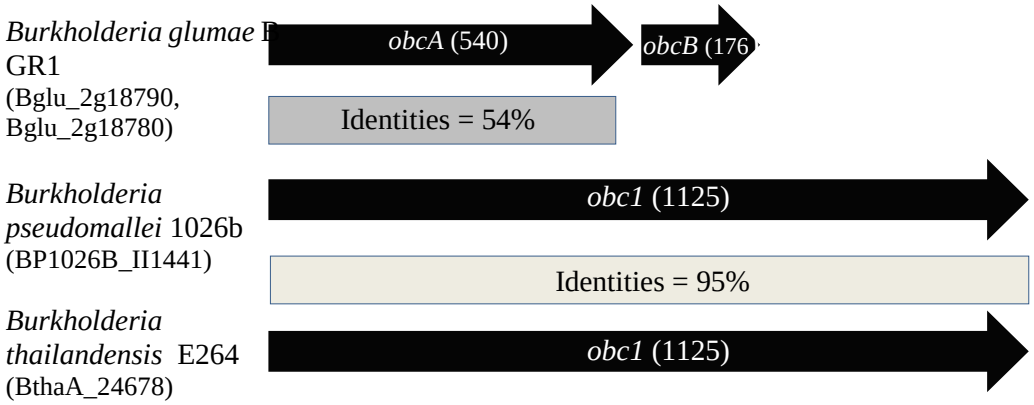
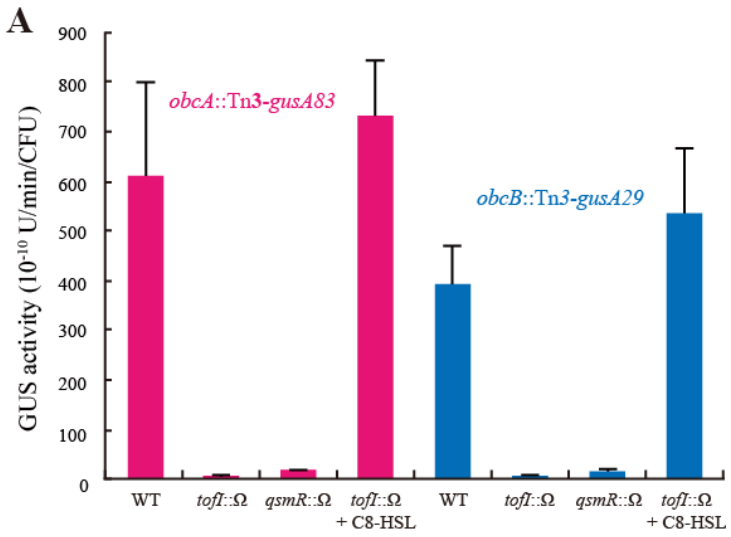
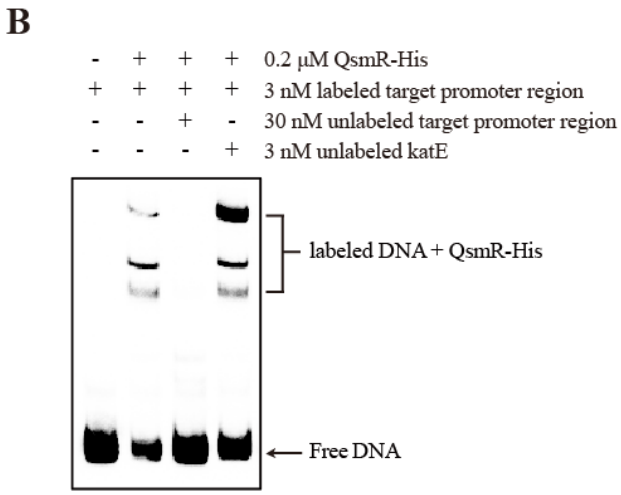

Burkholderia glumae BGR1
(Bglu_2g18790, Bglu_2g18780)
obcA (540)
obcB (176
Identities = 54%
Burkholderia pseudomallei 1026b
(BP1026B_II1441)
obc1 (1125)
Identities = 95%
Burkholderia thailandensis E264
(BthaA_24678)
obc1 (1125)

Figure 8. Regulation of *obcAB* genes by QsmR in *B. glumae*. (*A*) Expression of a chromosomal *obcAB-gusA* transcriptional fusion in *B. glumae* requires TofI and QsmR. Where indicated, synthetic C8-HSL was added to a final concentration of 1 μM. (*B*) Electrophoretic mobility shift assay (EMSA) indicating binding of QsmR-His to the *obc* promoter region. For the EMSA, 0.2 μM QsmR-His, 3 nM labeled target promoter DNA, 30 nM unlabeled target promoter DNA, and 3 nM unlabeled *katE* promoter DNA were used. Error bars represent the error ranges of triplicate experiments.


A
GUS activity (10-10 U/min/CFU)
900
800
700
600
500
400
300
200
100
0
obcA::Tn3-gusA83
obcB::Tn3-gusA29
WT
tofI::Ω
qsmR::Ω
tofI::Ω + C8-HSL
WT
tofI::Ω
qsmR::Ω
tofI::Ω + C8-HSL


B
- + + + 0.2 μM QsmR-His
+ + + + 3 nM labeled target promoter region
- - + - 30 nM unlabeled target promoter region
- - - + 3 nM unlabeled katE
labeled DNA + QsmR-His
Free DNA

Burkholderia glumae 균의 quorum sensing 의존 단백질체 분석과 생장 정지기 스트레스에 대한 예측

구은혜

초 록

세균성벼알마름병은 그람음성세균 *Burkholderia glumae* 에 의해 일어나는 병으로서 우리나라와 일본, 동남아시아를 비롯해 미국 벼 재배 지역에서 심각하게 발병되는 병이다. 최근 우리나라 여름이 아열대성 기후와 유사해지면서 본 병의 발생이 확산되고 있다. 이 병은 세균성벼알마름병균이 분비하는 toxoflavin이 독소로서 주요 병원성 인자 역할을 하고, 이 독소 생합성 유전자와 그들의 발현 조절에 대한 연구가 이루어졌다.

세균성벼알마름병균은 LuxI-LuxR type의 quorum sensing (QS) system을 가지고 있으며 TofI에 의해 만들어지는 *N*-octanoyl homoserine lactone (C8-HSL)과 signal receptor인 TofR을 사용하여 toxoflavin 생합성 유전자의 발현 뿐만 아니라 IclR-type transcriptional regulator인 *qsmR* 유전자의 발현을 activation한다. 이 균에서 QS가 병원성에 중요한 역할을 하기 때문에 QS에 의해 의존적인 단백질체를

2-dimensional gel electrophoresis와 ESI-MS/MS방법으로 분석하였다. 기존에 알려진 QS에 의존적인 단백질을 포함하여 총 79 개의 단백질이 wild-type BGR1과 *tofI* mutant BGS2에서 발현의 차이를 보였다. 이 중에서 59 개의 단백질은 세포 밖 (extracellular fraction)에서 발견되었으며 20 개는 세포질 내 (cellular fraction) 에 존재하였다. Protease, lipase, phosphatase를 포함하는 34 개의 extracellular 단백질은 type II secretion system (T2SS)를 통과하여 방출되었다. Real-time RT-PCR 분석을 통해 49 개의 extracellular 단백질과 13개의 cellular 단백질에 해당하는 유전자의 발현이 QS에 의해 조절받는 것이 확인되어 transcriptional level 에서도 이 들 유전자들의 발현이 QS에 의해 조절받는 것을 알았다. 세 개의 독립된 transcriptional units과 12 개의 general secretion pathway (*gsp*) 유전자로 구성된 T2SS는 QS에 의해 조절되고 특히 QsmR이 *gsp* 유전자의 발현을 직접 조절하였다. T2SS 결핍 mutants는 벼 이삭에 병을 wild type 보다 적게 냈으므로 T2SS에 의존적인 extracellular 단백질이 세균성벼알마름병균의 병원성에 중요한 역할을 한다는 것을 확인하였다.

본 연구에서는 세균성벼알마름병균의 QS가 이 세균의 T2SS를 통한 단백질 방출뿐만 아니라 세균의 생장 곡선에서 정지기의 스트레스를 예측하는데 주요 역할을 함을 밝혔다. Acyl-homoserine lactone에 의한 QS는 많은 *Proteobacteria* 종에서 다양한 활동을 조절하는데, 주로 방출되거나 분비되는 공공재 (公共財, public goods)를 생산하는 데 필요한 유전자의 발현을 조절한다. QS를 통한 공공재의 조절은

진화학적으로 봤을 때 집단의 생존을 위한 적응도 (fitness)에 유리함을 제공한다. QS의 다른 잠재적 역할은 과밀성 (overcrowding)을 예측할 수 있다는 가설하에 연구를 진행하였다. QS가 결핍된 세균성벼알마름병균을 배양하면 생장 정지기에서 알칼리 스트레스로 인해 세포가 사멸하게 되는데, 이 원인은 아미노산을 탄소원으로 사용함으로서 발생하는 암모니아가 배양액을 알칼리화 함으로서 일어나는 현상이었다. 알칼리화된 배양액은 세균이 분비하는 oxalate에 의해 중성화 되는데 이 과정에서 QS가 알칼리 스트레스를 예측하고 oxalate가 QS에 의존적으로 생합성되고 분비되어 세균 집단이 사멸하지 않도록 하였다. 이 연구 결과는 밀도인식 기작이 세균 세포 대사를 바꾸어 집단이 공유할 수 있는 자원 또는 공공재인 oxalate를 만들어 생장 정지기 또는 세균 집단이 가지는 용적 (capacity)를 예측하는 수단으로 사용됨을 실험적으로 처음 증명하였다.

주요어 : 세균성벼알마름병, 밀도인식, 단백질체학, 생장 정지기 스트레스, 옥살레이트

학번 : 2006-23279

맺으며..

지난 6년 반의 시간을 되돌아보며 지금의 저를 있게 해주신 많은 분들께 감사의 인사를 드리려합니다. 집을 떠나 모든 것이 낯선 서울 생활을 시작한 저에게 실험실 선배들은 또 다른 가족과도 같았습니다. 아무것도 모르던 첫 해에 기본적인 실험부터 생활까지 잘 가르쳐주고 챙겨주신 연화언니, 옥희언니, 진우오빠, 종희오빠, 용성오빠, 훈이오빠, 홍섭오빠, 재은언니, 나연언니, 경주오빠, 희진이에게 감사의 마음을 전합니다. 그리고 해를 거듭하면서 생긴 후배들에게 제가 받았던 관심과 사랑을 다 전해주지 못 한 것 같아 미안한 마음이 들고, 까딸스럽고 쌀쌀맞은 저를 이해해주고 졸업을 누구보다도 기쁘게 축하해준 세경이, 혜윤이, 지영언니, 현희, 혜성이, 운정씨, 민정언니에게도 그동안 표현하지 못 했던 고마움을 전합니다. 무엇보다 지도교수님이신 황인규 선생님의 지도 하에 정확하고 흐트러짐없이 연구하는 법을 체득하였고 딸처럼 아껴주셔서 사랑받으며 한 연구자로 잘 성장할 수 있었습니다.

지나간 일들을 생각해보면 순조로웠을 때보다 그렇지 않았던 때가 더 많았습니다. 열심히 일했던 실험 결과들에서 어떤 의미도 찾을 수 없고 다른 주제로도 바꿔보았지만 비슷한 부분에서 또 막혀 더이상 앞으로 나아갈 수 없었을 때는 절망도 많이 했었고 포기하려고도 했었습니다. 하지만 지도교수님을 믿고 끈기와 노력으로 견뎌 내 고진감래를 경험하였습니다. 이와 같은 경험으로 연구에서의 성과 뿐만 아니라 예전의 나를 놓고 새로운 나를 찾는 공부를 하여 성숙한 모습을 갖추게 된 것

같습니다. 박사학위를 받은 후의 삶이 더 중요하다고 하신 선생님의 말씀을 마음 깊이 새기고 학자로서 옳곧게 살아가도록 노력하겠습니다.

논문 심사 과정에서 위원장을 맡아주신 이인원 교수님의 세심하며 명철한 평가는 앞으로 제가 개선해 나가야할 과제가 되었으며 선생님께 진심으로 감사 드립니다. 바쁘신 와중에 심사에 참여해주신 이용환 교수님과 다른 과 학생임에도 흔쾌히 심사 위원이 되어주신 이상기 교수님, 본 논문 심사를 위해 대전에서 여러 차례 왕래해주신 문제선 박사님께도 깊은 감사를 드립니다. 그리고 식물미생물 전공에 김영호 교수님, 박은우 교수님, 김국형 교수님, 가종억 교수님께도 감사의 말씀을 드립니다.

마지막으로 묵묵히 지켜봐주고 힘들 때 든든한 지원군이 되어준 부모님과 지혜, 지영이, 원불교 인연들, 02학번 동기들에게도 감사의 마음을 전합니다.