A conserved two-component regulatory system, PidS/PidR, globally regulates pigmentation and virulence-related phenotypes of *Burkholderia glumae*

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SUMMARY

Burkholderia glumae is a rice pathogenic bacterium that causes bacterial panicle blight. Some strains of this pathogen produce dark brown pigments when grown on casamino-acid peptone glucose (CPG) agar medium. A pigment-positive and highly virulent strain of *B. glumae*, 411gr-6, was randomly mutagenized with mini-Tn5gus, and the resulting mini-Tn5gus derivatives showing altered pigmentation phenotypes were screened on CPG agar plates to identify the genetic elements governing the pigmentation of *B. glumae*. In this study, a novel two-component regulatory system (TCRS) composed of the PidS sensor histidine kinase and the PidR response regulator was identified as an essential regulatory factor for pigmentation. Notably, the PidS/PidR TCRS was also required for the elicitation of the hypersensitive response on tobacco leaves, indicating the dependence of the hypersensitive response and pathogenicity (Hrp) type III secretion system of *B. glumae* on this regulatory factor. In addition, *B. glumae* mutants defective in the PidS/PidR TCRS showed less production of the phytotoxin, toxoflavin, and less virulence on rice panicles and onion bulbs relative to the parental strain, 411gr-6. The presence of highly homologous PidS and PidR orthologues in other *Burkholderia* species suggests that PidS/PidR-family TCRSs may exert the same or similar functions in different *Burkholderia* species, including both plant and animal pathogens.

INTRODUCTION

Burkholderia glumae is the major causative agent of bacterial panicle blight (BPB) in rice (Ham *et al.*, 2011). Outbreaks of BPB have resulted in severe yield losses in the southern USA, including Texas, Arkansas and Louisiana, and damage from *B. glumae* infection is also a growing problem in east and south-east Asia and Central America (Ham *et al.*, 2011). Several studies on *B. glumae* have revealed that toxoflavin (Kim *et al.*, 2004), lipase activity (Devescovi *et al.*, 2007) and the motility driven by flagella (Kim *et al.*, 2007) are crucial for the pathogenicity of this pathogen. The

quorum-sensing (QS) system mediated by the LuxI and LuxR homologues, TofI and TofR respectively, is required for the production of these virulence factors, indicating that the TofI/TofR QS system is a crucial global regulatory factor for the pathogenic behaviour of *B. glumae* (Devescovi *et al.*, 2007; Kim *et al.*, 2004, 2007). Additional regulatory factors of *B. glumae* for virulence include ToxJ and ToxR, which positively regulate the expression of the genes for toxoflavin biosynthesis and transport, and QsmR, which activates the genes for flagellar biogenesis (Kim *et al.*, 2007, 2009). Previous studies have demonstrated that the genes encoding these regulatory factors are dependent on the TofI/TofR QS system for their expression, constituting the regulatory cascades for toxoflavin biosynthesis (TofI/TofR → ToxJ → ToxR → toxoflavin biosynthesis genes) and flagellar biogenesis (TofI/TofR → QsmR → FlhDC → flagellar biogenesis genes) (Kim *et al.*, 2004, 2007).

Recently, we discovered that some *B. glumae* strains produce dark pigments on casamino-acid peptone glucose (CPG) agar medium (Schaad *et al.*, 2001) (H. S. Karki and J. H. Ham, unpublished) (Fig. 2A). In our preliminary analyses with gel filtration chromatography and sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), at least three different pigment compounds were detected from a pigment-producing *B. glumae* strain grown on CPG agar medium, but none resembled any known melanin-type pigment (B. S. Kim and J. H. Ham, unpublished). The chemical structures of these pigments and their roles in parasitic fitness are currently being studied with natural strains and artificial mutants of *B. glumae* showing differential phenotypes in pigmentation.

In this study, a highly virulent and pigment-producing strain, 411gr-6, was randomly mutagenized with mini-Tn5gus (Fouts *et al.*, 2002) in an attempt to identify the genetic elements required for the pigmentation of *B. glumae*. The mutants showing altered pigmentation compared with the parental strain were screened among the resultant random mini-Tn5gus derivatives of 411gr-6 grown on CPG agar medium plates, and their mutated genes were subsequently identified by sequencing the genomic regions flanking the inserted mini-Tn5gus. In this article, we report that a novel two-component regulatory system (TCRS) composed of the PidS sensor histidine kinase (SHK) and the PidR response regulator (RR) is essential for the pigmentation phenotype and is required for the full virulence of *B. glumae*. We also demonstrate

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that the same TCRS is required for the elicitation of the hypersensitive response (HR) on tobacco leaves by *B. glumae*, indicating that it is an essential regulatory factor for the function of the hypersensitive response and pathogenicity (Hrp) type III secretion system (T3SS) of this pathogen.

RESULTS

Identification of genes involved in the pigmentation of *B. glumae*

Among ~20 000 random mini-Tn5gus derivatives of *B. glumae* 411gr-6, six mutant derivatives were initially screened on the basis of their no- or reduced-pigmentation phenotypes on CPG agar medium plates. Two of these pigment-deficient mutant derivatives, LSUPB112 and LSUPB115, were found to be disrupted by the insertion of mini-Tn5gus in an open reading frame (ORF) encoding a putative SHK (Table 1 and Fig. 1). Additional mutants showing no

pigment production, LSUPB114 and LSUPB116, were found to have a mini-Tn5gus insertion in the ORFs encoding a putative 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19) and a putative 3-dehydroquinase synthase (EC 4.2.3.4), respectively (Table 1). Both of these enzymes are components of the shikimic acid pathway (Duncan *et al.*, 1984; Millar and Coggins, 1986), suggesting that this metabolic pathway produces important precursor(s) of the pigment compounds of *B. glumae*. In addition, two mutant derivatives, LSUPB118 and LSUPB119, produced smaller amounts of pigment than the parental strain 411gr-6, and their disrupted ORFs were predicted to encode a quinoprotein glucose dehydrogenase and a succinate dehydrogenase iron-sulphur subunit, respectively (Table 1).

In this study, LSUPB112 and LSUPB115, which contain a mini-Tn5gus insertion in a putative SHK gene, were chosen for further investigation among the initially screened mutants in order to determine the virulence-related functions of this newly found regulatory gene of *B. glumae*.

Table 1 Description of mutated genes in the mini-Tn5gus derivatives of *Burkholderia glumae* 411gr-6 that show pigment-deficient phenotypes.

Strain name	Gene ID of mutated gene	Gene name*	Putative product/function	Pigment production
LSUPB112/LSUPB115	bglu_1g00490	<i>pidS</i> (this study)	Sensor histidine kinase/signal perception and transduction	No
LSUPB114	bglu_1g08780	<i>aroA</i> (Duncan <i>et al.</i> , 1984)	3-Phosphoshikimate 1-carboxyvinyltransferase/shikimic acid pathway	No
LSUPB116	bglu_1g03040	<i>aroB</i> (Millar and Coggins, 1986)	3-Dehydroquinase synthase/shikimic acid pathway	No
LSUPB118	bglu_2g12650	<i>gdh</i> (Cleton-Jansen <i>et al.</i> , 1988)	Quinoprotein glucose dehydrogenase/glucose metabolism	Reduced
LSUPB119	bglu_2g08260	<i>sdhB</i> (Darlison and Guest, 1984)	Succinate dehydrogenase iron-sulphur subunit/tricarboxylic acid (TCA) cycle	Reduced

*All the listed gene names, except *pidS*, are from original studies with *Escherichia coli*.

Fig. 1 A physical map of the *pidS* and *pidR* genes and their clones. This diagram is based on the sequence information of the *Burkholderia glumae* BGR1 genome (GenBank: CP001503.2). Open reading frames (ORFs) are symbolized by rectangles and arrows, and their corresponding locus tags in the GenBank feature file of CP001503.2 are indicated above each ORF. Black triangles indicate the positions of mini-Tn5gus in the mutant strains, LSUPB112 and LSUPB115. Restriction sites in the map are represented by: Ba, *Bam*HI; H, *Hind*III; E, *Eco*RI; P, *Pst*I; N, *Not*I; M, *Mlu*I; S, *Sma*I; Bg, *Bgl*II; B, *Bam*HI; Sp, *Spe*I. Restriction sites used for the subcloning of *pidS* and *pidR* are shown in parentheses. pCL126 is a cosmid clone harbouring the *pidR/pidS* locus. The broad-host-range vectors for pPidRS-1 and pPidRS-2 are pBBR1MCS-5 (Gm^R) and pBBR1MCS-2 (Km^R) (Kovach *et al.*, 1994), respectively. The 0.5-kb upstream region of *pidR*, which is predicted to be the promoter region of the putative *pidRS* operon, is indicated as a light grey line. CA, catalytic and ATP binding domain; DHp, dimerization and histidine phosphotransfer domain; REC, receiver domain.

Identification of a novel TCRS that controls the pigmentation of *B. glumae*

In LSUPB112 and LSUPB115, mini-Tn5gus was inserted at different genomic locations within an ORF encoding a putative SHK gene, indicating that the two mini-Tn5 derivatives of *B. glumae* are two independent mutants of the same SHK gene (Fig. 1). This identified SHK gene corresponds to 'bglu_1g00490' of the BGR1 genome and forms a putative operon with the ORF located upstream which encodes a putative RR (bglu_1g00500) (Fig. 1). Based on the initially observed pigment-deficient phenotypes of LSUPB112 and LSUPB115, the putative genes for the SHK and RR were named *pidS* (*pigment-deficient SHK*) and *pidR* (*pigment-deficient RR*), respectively.

To determine the functionality of *pidR*, its ORF was disrupted through a single homologous recombination with its internal region (Fig. 1). The two derivatives of 411gr-6 generated from this procedure, LSUPB133 and LSUPB225 (Fig. 1 and

Table 4), also showed pigment-deficient phenotypes, like LSUPB112 and LSUPB115 (Fig. 2A). However, it cannot be ruled out that the pigment-deficient phenotypes of the *pidR* mutants are caused by a polar effect of the *pidR* mutation on the downstream gene, *pidS*. To determine whether *pidR* alone is required for pigment production, the *pidR* mutant, LSUPB225, was tested for complementation with plasmids carrying either *pidS* only (pPidS-1) or both *pidR* and *pidS* (pPidRS-1) (Fig. 1). As shown in Fig. 2D, pigmentation of LSUPB225 was restored by pPidRS-1, but not by pPidS-1, indicating that *pidR* is also required for pigmentation, like *pidS*. pPidS-1 carries a functional copy of *pidS* because it can restore pigmentation of the *pidS* mutants, LSUPB112 and LSUPB115 (Fig. 2D). Currently, the generation of a nonpolar *pidR* mutant by the precise deletion of *pidR* is also being undertaken to confirm the *bona fide* function of *pidR*. Taken together, these observations and experiments validate the essential role of the PidS/PidR TCRS in pigment production.

Fig. 2 Pigmentation and hypersensitive response (HR) phenotypes of *Burkholderia glumae* strains. (A) Pigmentation phenotypes of two virulent *B. glumae* strains, 411gr-6 and 336gr-1, and *pidS*⁻ (LSUPB112 and LSUPB115) and *pidR*⁻ (LSUPB133) derivatives of 411gr-6 on casamino-acid peptone glucose (CPG) agar plates. (B) Pigmentation phenotypes of *B. glumae* strains 411gr-6, LSUPB112 and LSUPB112 (pPidRS-1) on CPG agar plates. (C) HR elicitation phenotypes of *B. glumae* strains 411gr-6, LSUPB115, LSUPB115 (pPidRS-1) and LSUPB133 on a tobacco leaf. (D) Pigmentation phenotypes of 411gr-6 and its *pidR*⁻ (LSUPB225) and *pidS*⁻ (LSUPB112 and LSUPB115) derivatives (top), and of the *pidR*⁻ and *pidS*⁻ derivatives carrying pPidS-1 or pPidRS-1 (bottom). For (A), (B) and (D), freshly grown bacterial colonies were streaked on CPG agar plates, which were incubated at 30 °C, and photographed 48 h after inoculation. For (C), c. 2.5×10^8 cfu/mL of each *B. glumae* strain resuspended in 10 mM MgCl₂ were infiltrated into a fully expanded tobacco leaf, which was photographed 18 h after infiltration.

Table 2 A summary of the phenotypes observed for the characterization of the functions of *pidS* and *pidR*.

<i>Burkholderia glumae</i> strains	Pigmentation	Hypersensitive response elicitation	Toxoflavin	Virulence on rice	Virulence on onion
411gr-6	+	+	+	Virulent	Virulent
LSUPB112	–	–	Less	Less virulent	Less virulent
LSUPB115	–	–	Less	Less virulent	Less virulent
LSUPB133	–	–	Less	Not done	Less virulent
LSUPB112 (pPidRS-1)	+	+	Not done	Not done	Virulent
LSUPB115 (pPidRS-1)	+	+	Not done	Not done	Virulent
LSUPB133 (pPidRS-2)	+	Not done	Not done	Not done	Virulent
LSUPB225 (pPidRS-1)	+	Not done	Not done	Not done	Not done
LSUPB225 (pPidS-1)	–	Not done	Not done	Not done	Not done
LSUPB112 (pPidS-1)	+	Not done	Not done	Not done	Not done
LSUPB115 (pPidS-1)	+	Not done	Not done	Not done	Not done

Role of the *PidS/PidR* TCRS in HR elicitation on tobacco leaves and in other virulence-related phenotypes

To determine the role of this TCRS in bacterial virulence, the *pidS* mutants, LSUPB112 and LSUPB115, were tested for several virulence-related phenotypes, including toxoflavin production, lipase activity, flagellum-mediated motility, HR elicitation in tobacco plants and virulence in rice.

Remarkably, both LSUPB112 and LSUPB115 failed to elicit an HR on tobacco leaves and produced less toxoflavin (Table 2, Figs 2C and 3A) than the parental strain. Moreover, these mutant strains were significantly less virulent than the parental strain on rice panicles (Table 2 and Fig. 3B). However, these mutants did not show significant differences in lipase activity or flagellum-mediated motility (data not shown). Like the *pidS* mutants, the newly generated *pidR*[–] strain, LSUPB133, could not elicit an HR on tobacco leaves (Table 2 and Fig. 2C) and showed reduced toxoflavin production relative to the parental strain (Table 2). In the study of Gram-negative plant pathogenic bacteria, the ability of bacteria to elicit an HR on nonhost plant leaves, such as tobacco, is considered to be a hallmark of the presence of a functional T3SS (Alfano and Collmer, 2004; Collmer *et al.*, 2000). *Burkholderia glumae* is known to possess a functional T3SS, which is required for both HR elicitation on tobacco leaves and full virulence (Kang *et al.*, 2008). Thus, failure of HR elicitation by the *pidS* and *pidR* mutants strongly suggests that the *PidS/PidR* TCRS is also essential for the expression of T3SS genes in *B. glumae*. In addition, other virulence-related phenotypes of the *pidS* and *pidR* mutants suggest that this TCRS partially controls the genes for toxoflavin biosynthesis and contributes to the full virulence of this pathogen.

Complementation of *pidS* and *pidR* mutant phenotypes

As described in the Experimental Procedures section, functional *pidS* and *pidR/pidS* clones were constructed for genetic confirmation of the pigment-deficient phenotypes of the *pidS* and *pidR*

mutations (Fig. 1). The recovery of virulence-related phenotypes, including HR elicitation on tobacco leaves and virulence on onion bulb scales, was also tested for *pidR* and *pidS* mutants complemented with a *pidR/pidS* clone. The DNA constructs carrying both *pidR* and *pidS*, pPidRS-1 and pPidRS-2, were transconjugated into the *pidS* or *pidR* mutants of *B. glumae* through triparental mating (Figurski and Helinski, 1979). The resultant transconjugants, selected on Luria–Bertani (LB) agar containing nitrofurantoin and gentamycin (for pPidRS-1) or kanamycin (for pPidRS-2), showed restored functions in pigment production on CPG agar plates (Table 2 and Fig. 2B) and in HR elicitation in tobacco (Table 2 and Fig. 2C). In addition, complementation of the *pidS* and *pidR* mutants with pPidRS-1 resulted in a substantial increase in virulence in onion bulb assays (Table 2 and Fig. 3C), which were originally used to test the virulence of onion pathogenic *Burkholderia* species (Jacobs *et al.*, 2008). This assay system using onion bulbs is an excellent alternative method for the quantification of the virulence of *B. glumae* because virulent strains of *B. glumae* can also produce measurable maceration symptoms on onion bulbs, and their virulence on onion bulbs is highly correlated with their virulence on rice panicles (H. S. Karki and J. H. Ham, unpublished). The results of all the complementation tests conducted in this study clearly indicate that the observed phenotypes of *pidS* and *pidR* mutants are *bona fide*.

DISCUSSION

In this study, it was demonstrated that a novel TCRS, *PidS/PidR*, is an essential regulatory component of *B. glumae* for pigmentation on CPG agar medium, HR elicitation in tobacco and full virulence in rice and onion. At this time, little is known about the pigments produced by *B. glumae* 411gr-6. Products of the shikimic acid pathway may be important intermediates of the pigments because LSUPB114 and LSUPB116, which contain mini-Tn5*gus* insertions in ORFs encoding two putative metabolic enzymes for the shikimate pathway (3-phosphoshikimate 1-carboxyvinyltransferase and 3-dehydroquinate synthase respectively), failed to produce pigments like *pidS* mutant derivatives (Table 1). Meanwhile, two

Fig. 3 Phenotypes of *Burkholderia glumae* strains in toxoflavin production and virulence. (A) Toxoflavin production of *B. glumae* strains 411gr-6, LSUPB112 and LSUPB115 on King's B agar plates. Each error bar indicates the standard deviation from three replications. (B) Symptoms on rice panicles caused by *B. glumae* strains 411gr-6, LSUPB112 and LSUPB115. Numbers indicate the average scores of disease severity (DS) from 10 replications evaluated using a 0–9 scale at 10 days post-inoculation. On this scale, 0 denotes no symptoms and 9 indicates that more than 81% of the panicle is symptomatic. Photographs were taken at 10 days post-inoculation. Superscript letters indicate statistically significant differences ($P > 0.01$) among disease ratings. (C) Symptoms on onion bulb scales caused by *B. glumae* strains 411gr-6, LSUPB112, LSUPB115, LSUPB133, LSUPB112 (pPidRS-1), LSUPB115 (pPidRS-1) and LSUPB133 (pPidRS-2). Numerical values indicate the average macerated area (mm²) from three replications. The photograph was taken at 48 h post-inoculation at the time of symptom evaluation.

mutant derivatives of 411gr-6, LSUPB118 and LSU119, showed partial defects in pigmentation (Table 1). LSUPB118 has a mini-Tn5gus insertion in a *gdh* homologue encoding a quinoprotein glucose dehydrogenase, whereas LSUPB119 has an insertion in a *sdhB* homologue encoding a succinate dehydrogenase iron-sulphur subunit (Table 1). Interestingly, both quinoprotein glucose dehydrogenase and succinate dehydrogenase are involved in electron transport for oxidative phosphorylation and produce ubiquinol by reducing ubiquinone during their enzymatic reactions (Elias *et al.*, 2001; Hagerhall, 1997). We speculate that the reduction power created by these oxidoreductases might contribute to the pigment production in *B. glumae*.

Keith *et al.* (2007) reported recently that some strains of *B. cenocepacia*, which cause opportunistic infections in humans, produce a melanin-like pigment, and the production of this pigment is

dependent on *hpdD*, which encodes a 4-hydroxyphenylpyruvate deoxygenase (HppD). This indicates that homogentisate (HGA), which is synthesized by HppD, is an important precursor of the melanin-like pigment. In their study, the melanin-like pigment contributed to the attenuation of the oxidative burst in host cells by scavenging free radicals (Keith *et al.*, 2007). We do not yet know whether the pigments of *B. glumae* are related to this pigment produced by *B. cenocepacia*. We are currently characterizing the chemical structures and biological functions of the *B. glumae* pigments. Our preliminary data indicate that *B. glumae* 411gr-6 produces at least three different pigments (brown, purple and fluorescent compounds) (B. S. Kim and J. H. Ham, unpublished), that are probably involved in the tolerance to environmental stresses, including reactive oxygen species and ultraviolet light (H. S. Karki and J. H. Ham, unpublished). In addition, pigment-deficient

derivatives of 411gr-6 generated by mini-Tn5gus insertion show reduced virulence in rice, suggesting that pigmentation acts positively in parasitic fitness via increased tolerance to environmental stresses (H. S. Karki and J. H. Ham, unpublished). Nevertheless, it is unlikely that the pigments are required for the virulence on host plants, because several naturally avirulent strains isolated from rice fields also exhibit similar pigmentation phenotypes, whereas many virulent strains of *B. glumae* do not (H. S. Karki and J. H. Ham, unpublished). To determine whether the production of the *B. glumae* pigments is also dependent on HGA, we are currently generating *hppD* mutants of *B. glumae*. It is expected that these ongoing studies will elucidate the structural and functional characteristics of the *B. glumae* pigments and their relatedness to the pigments produced by other *Burkholderia* spp., such as *B. cenocepacia*.

HR elicitation by Gram-negative plant pathogenic bacteria in nonhost plants, such as tobacco, is a hallmark of a functional T3SS (Alfano and Collmer, 2004). The loss of ability to elicit HR in tobacco leaves caused by mutations in *pidS* and *pidR* indicates that the PidS/PidR TCRS is required for the expression of a functional T3SS in *B. glumae* (Fig. 2C). In many plant pathogenic bacteria having narrow host ranges, such as *Pseudomonas* spp. and *Xanthomonas* spp., T3SSs encoded by *hrp* (*hypersensitive response and pathogenicity*) genes are essential for both HR elicitation in nonhosts or resistant hosts and pathogenicity in susceptible hosts (Alfano and Collmer, 2004; Collmer *et al.*, 2000). However, T3SSs are frequently dispensable for bacterial infection and only contribute to full virulence in plant pathogenic bacteria, including *Pectobacterium* spp., which have broad host ranges and utilize extracellular enzymes secreted via a type II secretion system as primary virulence factors (Bauer *et al.*, 1994; Holeva *et al.*, 2004; Rantakari *et al.*, 2001). In this study, *pidS* and *pidR* mutants of *B. glumae* could not elicit an HR on tobacco leaves (Fig. 2C), but could produce symptoms in hosts at reduced levels (Fig. 3B,C). These results indicate that the Hrp T3SS of *B. glumae* is not essential for pathogenicity, even though it is required for full virulence. This speculation is consistent with a previous study in which a T3SS-deficient *B. glumae* mutant still produced symptoms in rice, although it was significantly less virulent than its parental strain (Kang *et al.*, 2008). In this regard, it is noteworthy that *B. glumae* also produces more important virulence factors, including the phytoxin, toxoflavin (Kim *et al.*, 2004), and lipase (Devescovi *et al.*, 2007). In *B. glumae*, the pathogenicity of T3SS-deficient mutants is retained, probably because of these other major virulence factors.

In prokaryotes, TCRSs play a pivotal role in signal perception and transduction for a wide range of cellular functions involved in metabolism, development and pathogenesis (Laub and Goulian, 2007). In plant pathogenic bacteria, several TCRSs are also known to have global effects on virulence. For example, GacS/GacA of *Pseudomonas syringae* controls the production of every known virulence factor, including coronatine, extracellular polysaccha-

rides (EPSs) and the T3SS and its effectors, via positive regulation of the QS system (Mole *et al.*, 2007). The HrpX/HrpY TCRSs control the genes encoding T3SSs in plant pathogenic bacteria belonging to *Enterobacteriaceae*, including *Erwinia amylovora* (Wei *et al.*, 2000) and *Pantoea stewartii* (Merighi *et al.*, 2003). RpfC/RpfG of *Xanthomonas campestris* is known to regulate multiple virulence factors, including extracellular enzymes and EPSs, via the degradation of the signal molecule 3',5'-cyclic diguanylic acid and the interconnection with the cell-to-cell communication mediated by diffusible signal factor (DSF) (Ryan *et al.*, 2006). Recently, it has been reported that the ColS/ColR TCRSs of *X. campestris* pv. *campestris* (Zhang *et al.*, 2008) and *X. citri* ssp. *citri* (Yan and Wang, 2011) play important roles in tolerance to environmental stresses, T3SS and virulence (specifically ColS_{XC1050}/ColR_{XC1049} of *X. campestris* and ColS_{XAC3249}/ColR_{XAC3250} of *X. citri*). Interestingly, PidR shows more than 31% amino acid sequence identity with both ColR RRs of the two *Xanthomonas* spp., whereas PidS shows less than 20% identity with the corresponding ColS sensor kinases (Table 3). The apparently similar functions between PidS/PidR and ColS/ColR TCRSs in virulence and the significant sequence homology between the PidR and ColR RRs strongly suggest that both TCRSs act on common regulatory pathways for bacterial pathogenesis in different host environments.

Remarkably, it was found that the orthologues of PidS and PidR are highly conserved among many *Burkholderia* spp. *Burkholderia gladioli*, another bacterium causing BPB in rice (Nandakumar *et al.*, 2009), contains PidR and PidS orthologues which show the highest homology to PidR and PidS (Table 3). In particular, the *B. glumae* and *B. gladioli* PidR amino acid sequences were identical to each other (Table 3). The next closest orthologues of PidS were present in *B. pseudomallei* and *B. mallei*, and showed more than 84% amino acid sequence identity to that of PidS (Table 3). In particular, the first 200 amino acids from the N-termini, which correspond to the signal input domain, showed higher homology to PidS (>92% identity) than to the remaining regions containing dimerization and histidine phosphotransfer (DHp) and catalytic and ATP binding (CA) domains. Similar patterns were also observed in other PidS orthologues of *Burkholderia* spp. In general, N-terminal signal input domains of different SHKs tend to be more variable in amino acid sequence because they are responsible for sensing different signals. Higher amino acid sequence homology among PidS and its orthologues at the N-terminal signal input domain strongly suggests that these SHKs recognize the same or similar signals and, further, have similar biological functions in different species of *Burkholderia*. Orthologues of PidR from other *Burkholderia* spp. showed even higher levels of similarity to PidR. In particular, the PidR orthologues of *B. pseudomallei* and *B. mallei* were almost identical (99.6% identity) to PidR (Table 3). Other PidR orthologues of *Burkholderia* spp. showed more than 96% identity, indicating that PidR and the PidR orthologues of *Burkholderia* spp. may exert a common regulatory func-

Table 3 Comparison of the PidS/PidR two-component regulatory system with the representative orthologues of *Burkholderia* spp. and with the known two-component regulatory systems of other plant pathogenic bacteria.

PidS (YP_002909958.1: 517 amino acids)			PidR (YP_002909959.1: 230 amino acids)		
Protein ID	Organism	Amino acid sequence identity (%) [*]	Protein ID	Organism	Amino acid sequence identity (%)
YP_004358720.1	<i>B. gladioli</i> BSR3	90.6	YP_004358721.1	<i>B. gladioli</i> BSR3	100
YP_001068298.1	<i>B. pseudomallei</i> 1106a	84.3	YP_001068297.1	<i>B. pseudomallei</i> 1106a	99.6
YP_104430.1	<i>B. mallei</i> ATCC23344	84.2	YP_104431.1	<i>B. mallei</i> ATCC23344	99.6
ZP_02465317.1	<i>B. thailandensis</i> MSMB43	83.9	ZP_02465316.1	<i>B. thailandensis</i> MSMB43	99.6
ZP_02357567.1	<i>B. oklahomensis</i> EO147	83.6	ZP_02357566.1	<i>B. oklahomensis</i> EO147	99.6
ZP_02890082.1	<i>B. ambifaria</i> IOP40-10	82.9	ZP_02890081.1	<i>B. ambifaria</i> IOP40-10	98.7
YP_002229170.1	<i>B. cenocepacia</i> J2315	82.9	YP_002229171.1	<i>B. cenocepacia</i> J2315	98.7
ZP_02380348.1	<i>B. ubonensis</i> Bu	82.7	YP_001578267.1	<i>B. multivorans</i> ATCC17616	98.7
NP_643557.1 (ColS _{XAC3249})	<i>Xanthomonas citri</i> ssp. <i>citri</i> 306	19.4	NP_643558.1 (ColR _{XAC3250})	<i>Xanthomonas citri</i> ssp. <i>citri</i> 306	31.9
YP_242140.1 (ColS _{XC1050})	<i>Xanthomonas campestris</i> pv. <i>campestris</i> 8004	18.3	YP_242139.1 (ColR _{XC1049})	<i>Xanthomonas campestris</i> pv. <i>campestris</i> 8004	31.5
AAD24682.1 (HrpX)	<i>Erwinia amylovora</i> Ea321	17.4	AAD24683.1 (HrpY)	<i>Erwinia amylovora</i> Ea321	19.1
AAG01454.2 (HrpX)	<i>Pantoea stewartii</i> ssp. <i>stewartii</i> SS104	16.8	AAG01455.1 (HrpY)	<i>Pantoea stewartii</i> ssp. <i>stewartii</i> SS104	16.0

^{*}The Needleman–Wunsch algorithm (Needleman and Wunsch, 1970) was implemented to obtain the amino acid sequence identity values using the EMBOSS web tool with default settings (matrix, Blosum62; open gap penalty, 10.0; gap extension penalty, 0.5).

tion (Table 3). It would be very interesting to investigate whether the mutation of *pidS* or *pidR* orthologues also results in impaired T3SS function and reduced virulence in animal pathogenic *Burkholderia* spp., such as *B. mallei* and *B. pseudomallei*. If the regulatory mechanisms and virulence function of PidS and PidR proteins are conserved among pathogenic *Burkholderia* spp., further in-depth research on the PidS/PidR TCRS of *B. glumae* would provide valuable information for the study of animal/human pathogenic *Burkholderia* spp., which is often hindered by strict legal barriers and limitations.

Two approaches are currently being undertaken or planned for further characterization of the regulatory function of the PidS/PidR TCRS in *B. glumae*. First, the expression of known virulence genes and their regulatory genes is being tested in the presence or absence of *pidS* and *pidR* to determine the virulence genes under the control of this TCRS and the regulatory hierarchy between this novel regulatory system and other known regulatory systems. In our preliminary experiments, for example, expression of *tofl* for QS was not changed significantly in a *pidS* mutant background, indicating that the TofI/TofR QS system of *B. glumae* is independent or upstream of the PidS/PidR TCRS (H. S. Karki and J. H. Ham, unpublished). We are currently examining the expression of *pidRS* in *tofl*[−] and *toflR*[−] backgrounds to determine the dependence of the PidS/PidR TCRS on the TofI/TofR QS system. Second, RNA-seq and ChIP-seq using a high-throughput DNA sequencing technique are planned for genome-wide identification of the PidS and PidR regulons. These studies will allow us to better understand the regulatory functions of the PidS/PidR TCRS.

Conclusively, we have demonstrated that the newly discovered PidS/PidR TCRS is required for the pigmentation of *B. glumae*

under a certain nutritional condition, growth on CPG agar media, and for HR elicitation on tobacco leaves by this bacterium. The PidS/PidR TCRS has also been shown to contribute to the virulence of *B. glumae* in virulence assays on rice panicles and onion bulbs. *pidS* and *pidR* orthologues are highly conserved among other *Burkholderia* spp., suggesting that they may play similar roles in bacterial pathogenesis. Further study on this new regulatory factor will expand our knowledge on the global regulatory systems of *B. glumae* and other important *Burkholderia* spp.

EXPERIMENTAL PROCEDURES

Bacterial cultures and DNA manipulation

The bacterial strains and plasmids used in this study are listed in Table 4. *Escherichia coli* and *B. glumae* strains were routinely grown in LB medium (Sambrook, 2001) at 37 °C. King's B (KB) and CPG agar media (Schaad *et al.*, 2001) were used to test toxoflavin and pigment production by *B. glumae*, respectively. Antibiotics were included in the media as necessary at the following concentrations: ampicillin (100 µg/mL), gentamycin (20 µg/mL), kanamycin (50 µg/mL) and nitrofurantoin (100 µg/mL).

Standard protocols (Sambrook, 2001) were used for general DNA manipulation procedures, including extraction, restriction enzyme digestion, ligation, polymerase chain reaction (PCR) and agarose gel electrophoresis. DNA sequencing was performed by either the DNA sequencing facility (GeneLab) at the LSU School of Veterinary Medicine (Baton Rouge, LA, USA) or Macrogen, Inc. (Seoul, Korea). Oligonucleotides for PCR and DNA sequencing were purchased from Bioneer, Inc. (Alameda, CA, USA). Transformation of bacterial cells with plasmid DNA was performed through either electroporation at 200 Ω/1.5 kV using a GenePulser (Bio-Rad Laboratories, Hercules, CA, USA) or triparental mating (Figurski and Helinski, 1979).

Table 4 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
<i>Burkholderia glumae</i>		
336gr-1	A virulent strain showing a pigment-deficient phenotype, Nit ^R	This study
411gr-6	A virulent strain showing a pigment-proficient phenotype, Nit ^R	This study
LSUPB112	A <i>pidS</i> ::mini-Tn5gus derivative of 411gr-6, Nit ^R , Km ^R	This study
LSUPB115	A <i>pidS</i> ::mini-Tn5gus derivative of 411gr-6, Nit ^R , Km ^R	This study
LSUPB133	A <i>pidR</i> ::pKNOCK _{Gm} derivative of 411gr-6, Nit ^R , Gm ^R	This study
LSUPB225	A <i>pidR</i> ::pKNOCK _{Km} derivative of 411gr-6, Nit ^R , Km ^R	This study
<i>Escherichia coli</i>		
DH5α	<i>SupE44</i> <i>DlacU169</i> (f80 <i>lacZDM15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Life Technologies
S17-1 λpir	<i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR</i> [res- mod+][RP4::2-Tc::Mu-Km::Tn7] λ <i>pir</i> phage lysogen	(Simon <i>et al.</i> , 1983)
XL1-Blue MR	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i>	Stratagene
Plasmids		
pUT::miniTn5gus	A suicide vector carrying mini-Tn5gus, Ap ^R , Km ^R	(Fouts <i>et al.</i> , 2002; de Lorenzo <i>et al.</i> , 1990)
pKNOCK _{Gm}	A suicide vector, R6K <i>ori</i> , Gm ^R	(Alexeyev, 1999)
pKNOCK _{Km}	A suicide vector, R6K <i>ori</i> , Km ^R	(Alexeyev, 1999)
pBBR1MCS-2	A broad-host-range vector, Km ^R	(Kovach <i>et al.</i> , 1995)
pBBR1MCS-5	A broad-host-range vector, Gm ^R	(Kovach <i>et al.</i> , 1995)
SuperCos1	A cosmid vector, Ap ^R , Km ^R	Stratagene
pSC-A-amp/kan	A PCR cloning vector, Ap ^R , Km ^R	Stratagene
pCL126	A cosmid clone harbouring the <i>pidR/pidS</i> locus, Ap ^R	This study
pPidRi-1	A clone of <i>pidR</i> internal region in pKNOCK _{Gm} , Gm ^R	This study
pPidRi-2	A clone of <i>pidR</i> internal region in pKNOCK _{Km} , Km ^R	This study
pPidRS-1	A subclone of pCL126 for the 3.9-kb <i>HindIII/BglII</i> fragment harbouring both <i>pidR</i> and <i>pidS</i> in pBBR1MCS-5, Gm ^R	This study
pPidRS-2	A subclone of pCL126 for the 3.9-kb <i>HindIII/BglII</i> fragment harbouring both <i>pidR</i> and <i>pidS</i> in pBBR1MCS-2, Km ^R	This study
pPidS	A <i>pidS</i> clone in pBBR1MCS-5 carrying the 3.3-kb <i>EcoRI/SpeI</i> fragment from pPidRS-2, Gm ^R	This study
pPidS-1	A derivative of pPidS added with the 0.5-kb upstream region of <i>pidR</i> , Gm ^R	This study

Construction of a *B. glumae* cosmid library

A cosmid library of *B. glumae* was constructed for our standard virulent strain, 336gr-1, using the SuperCos1 cosmid vector kit (Stratagene, La Jolla, CA, USA) following the manufacturer's protocol. *Escherichia coli* XL1-Blue MR cells carrying individual cosmid clones of the library were maintained in LB broth or agar containing ampicillin.

Random mutation of *B. glumae* with mini-Tn5gus

Overnight cultures of *B. glumae* 411gr-6 and *E. coli* S17-1 λpir (pUT::mini-Tn5gus) were mixed in a 3 : 1 ratio (v/v), and 1 mL of the bacterial mixture was centrifuged at *c.* 10 000 *g* for 1 min in a microcentrifuge tube. Following centrifugation, the supernatant was discarded and the pellet was dissolved in 50 µL LB broth, spotted onto LB plates and incubated overnight at 30 °C. The mated bacteria were then resuspended in 1 mL of LB broth, and 100-µL aliquots of the bacterial suspension were spread on CPG agar plates containing nitrofurantoin and kanamycin. After 2 days of incubation at 30 °C, pigment-deficient mutants were screened on the basis of their pigmentation phenotypes on CPG agar medium.

Identification of genes disrupted by mini-Tn5gus

The flanking sequences of mini-Tn5gus integrated into the mutant genomes were amplified following the method developed by Kwon and Ricke (2000). Briefly, the Y-linker having a cohesive end compatible to *NlaIII* restriction sites was made by annealing two oligonucleotides,

5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG-3' and 5'-TGTCCTCCGTACATCGTTAGAACTACTCGTACCATCCACAT-3'. This Y-linker was then ligated to the genomic DNA of a mutant digested with *NlaIII*. The ligated DNA was subjected to PCR with the Y-linker primer, 5'-CTGCTCGAATTCAAGCTTCT-3', and the Tn5 specific primer, 5'-GGCCAGATCTGATCAAGAGA-3', under the following reaction conditions: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 1 min and 70 °C for 1 min, and a final extension at 70 °C for 5 min. A Y-linker for *PstI* digestion made with two oligonucleotides, 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACTGCA-3' and 5'-GTGTCCTCCGTCATCGTTAGAACTACTCGTACCATCCACAT-3', was used for mutants that could not be identified with the *NlaIII* digestion scheme. The amplified PCR products from this procedure were purified using a QuickClean 5M PCR Purification Kit (GenScript, Piscataway, NJ, USA) and sequenced for the identification of genes mutated by mini-Tn5gus insertion.

Generation of *pidR*::pKNOCK mutants (LSUPB133 and LSUPB225)

An internal region of *pidR* was initially amplified using DNA primers, 5'-AAGTGCCTCAGATGGTCT-3' and 5'-AACTCGTGATCCTCGACCTG-3', and cloned into suicide vectors, pKNOCK_{Gm} and pKNOCK_{Km} (Alexeyev, 1999), generating pPidRi-1 and pPidRi-2, respectively. *Escherichia coli* S17-1 λpir (Simon *et al.*, 1983) was used to maintain pKNOCK vectors and pPidRi-1 and pPidRi-2. pPidRi-1 and pPidRi-2 were then transconjugated into *B. glumae* 411gr-6 via triparental mating with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979). *pidR* mutants were selected on LB agar medium containing nitrofurantoin and gentamycin

(for LSUPB133) or nitrofurantoin and kanamycin (for LSUPB225). The mutation of *pidR* in LSUPB133 and LSUPB225 was confirmed by diagnostic PCR (data not shown).

Quantification of toxoflavin

The extraction and quantification of toxoflavin were performed following the protocols developed by Iiyama *et al.* (1995) with some modifications. Briefly, an overnight culture of *B. glumae* in LB was washed twice with an equal volume of fresh LB broth and resuspended in 1/10 volume of LB broth. Then, 20 µL of the bacterial suspension ($c. 5 \times 10^8$ cells) was spread on a KB agar plate, followed by incubation at 37 °C for 48 h. Bacterial cells grown on the KB agar plates were removed by flooding the plates with sterile H₂O, and the remaining KB agar media were cut into small pieces. Toxoflavin diffused in the chopped KB agar media was extracted with an equal volume of chloroform. After the chloroform had been evaporated completely in a fume hood, the residue was dissolved in 1 mL of aqueous 80% methanol and diluted five times in distilled water. The absorbance was measured at 260 nm for each sample using a spectrophotometer (Biomate 3, Thermo Scientific, Pittsburgh, PA, USA).

HR elicitation

Burkholderia glumae cells grown on LB agar medium overnight at 37 °C were resuspended in 10 mM MgCl₂ and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.5 [$c. 2.5 \times 10^8$ colony-forming units (cfu/mL)]. The bacterial suspension was infiltrated with a needle-less syringe into the fully expanded leaves of 10–12-week-old tobacco plants grown in a glasshouse. HR was observed 18 h after infiltration.

Virulence assay with rice panicles

Bacterial suspensions for inoculation were prepared with the same method as used for HR tests, except that the bacterial cells were resuspended in sterile tap water instead of 10 mM MgCl₂ and their concentrations were adjusted to OD₆₀₀ = 0.1 ($c. 5 \times 10^7$ cfu/mL). The rice variety Trenasse (*Oryza sativa* cv. Trenasse) was grown in a glasshouse and sprayed with the bacterial suspensions at the 20%–30% heading stage. Two days after the first inoculation, a second inoculation was made in the same way. The disease severity was evaluated 10 days after the first inoculation. The disease severity on rice panicles was determined by the following scale: healthy panicle, 0; 1%–10% symptomatic area, 1; 11%–20% symptomatic area, 2; 21%–30% symptomatic area, 3; 31%–40% symptomatic area, 4; 41%–50% symptomatic area, 5; 51%–60% symptomatic area, 6; 61%–70% symptomatic area, 7; 71%–80% symptomatic area, 8; >81% symptomatic area, 9.

Virulence assay with onion bulb scales

Virulence phenotypes of *B. glumae* strains were also determined using the onion assay method developed by Jacobs *et al.* (2008). Briefly, scales of onion bulbs were cut into pieces ($c. 10$ cm²) with a sterile razorblade and placed in a wet chamber. Bacterial suspensions of *B. glumae* strains were prepared with the same method as used for HR tests, except that the

bacterial concentrations were adjusted to OD₆₀₀ = 0.2 ($c. 1 \times 10^8$ cfu/mL). Five microlitres of the suspension containing $c. 5 \times 10^5$ cells were applied to a $c. 2$ -mm slit made in the centre of each onion scale ($c. 10$ cm²) with a micropipette tip, and the inoculated onion scales were incubated in a wet chamber at 30 °C for 48 h. The virulence of each *B. glumae* strain was quantified by measuring the macerated area.

DNA constructs for genetic complementation tests

To generate pPidRS-1/pPidRS-2, a cosmid library of the genome of *B. glumae* 336gr-1, another highly virulent *B. glumae* strain, was screened by PCR using a *pidS* specific primer set, 5'-GTTGTCCTCCACCAGATCT-3' and 5'-CTGTCGAACAGTTGCTGTC-3', to identify cosmid clones containing *pidS*. One of the screened cosmid clones, pCL126, was digested with *Hind*III and *Bgl*II to obtain the 3.9-kb fragment that contains both *pidR* and *pidS*. This fragment was then subcloned into a broad-host-range vector, pBBR1MCS-5 or pBBR1MCS-2 (Kovach *et al.*, 1995), generating pPidRS-1 or pPidRS-2, respectively (Table 4 and Fig. 1).

To generate pPidS-1, pPidRS-2 was digested with *Eco*RI and *Spe*I to obtain the 3.3-kb fragment that included *pidS* but not *pidR*. This fragment was subcloned into pBBR1MCS-5 using the same restriction sites, generating pPidS. To ensure the expression of *pidS* in pPidS, the *cis* elements that may be required for the transcription of the predicted *pidRS* operon were placed in front of *pidS* with the following procedure. The 507-bp upstream region of the *pidR* coding sequence was amplified with the primer set, 5'-ACTAGGACAAACCCGTAG-3' and 5'-GGATCCGAAACCTGCTTGTC-3', and subsequently cloned into pSC-A-amp/kan using the StrataClone PCR Cloning Kit (Stratagene). The resultant PCR clone was digested with *Eco*RI and the *Eco*RI-cut fragment containing the *pidR* upstream sequence was then ligated to the *Eco*RI-cut pPidS, generating pPidS-1 (Fig. 1). The correct orientation of the *pidR* upstream region in front of *pidS* in pPidS-1 was confirmed by a series of diagnostic restriction digestions (data not shown).

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