Identification of the Regulatory Components Mediated by the Cyclic di-GMP Receptor Filp and Its Interactor PilZX3 and Functioning in Virulence of *Xanthomonas oryzae* pv. *oryzae*

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The degenerate GGDEF/EAL domain protein Filp was previously shown to function as a cyclic di-GMP (c-di-GMP) signal receptor through its specific interaction with an atypical PilZ domain protein PilZX3 (formerly PXO_02715) and that this interaction is involved in regulating virulence in *Xanthomonas oryzae* pv. *oryzae*. As a step toward understanding the regulatory role of Filp/PilZX3-mediated c-di-GMP signaling in the virulence of *X. oryzae* pv. *oryzae*, differentially expressed proteins (DEPs) downstream of Filp/PilZX3 were identified by isobaric tagging for relative and absolute quantitation (iTRAQ). A total of 2,346 proteins were identified, of which 157 displayed significant differential expression in different strains. Western blot and quantitative reverse transcription-PCR analyses showed that the expression of HrrP (histidine kinase-response regulator hybrid protein), PhrP (PhoPQ-regulated protein), ProP (prophage Lp2 protein 6) were increased in the Δ filp, Δ pilZX3, and Δ filp Δ pilZX3 mutant strains, while expression of CheW1 (chemotaxis protein CheW1), EdpX2 (the second EAL domain protein identified in *X. oryzae* pv. *oryzae*), HGdpX2 (the second HD-GYP domain protein identified in *X. oryzae* pv. *oryzae*) was decreased in all mutant strains compared with that in the wild type, which was consistent with the iTRAQ data. Deletion of the *hrrP* and *proP*

genes resulted in significant increases in virulence, whereas deletion of the *cheW1*, *hGdpX2*, or *tdrX2* genes resulted in decreased virulence. Enzyme assays indicated that EdpX2 and HGdpX2 were active phosphodiesterases (PDEs). This study provides a proteomic description of putative regulatory pathway of Filp and PilZX3 and characterized novel factors that contributed to the virulence of *X. oryzae* pv. *oryzae* regulated by c-di-GMP signaling.

Keywords: cyclic di-GMP receptor, degenerated PilZ domain protein, relative and absolute quantitation (iTRAQ), virulence, *Xanthomonas oryzae* pv. *oryzae*

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The raw MS proteomics data have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository under the dataset identifier PXD015734.

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Cyclic di-GMP (c-di-GMP) is a common bacterial second messenger that participates in the control of a variety of functions, including flagellar motility, biofilm formation, cell cycle, lifestyle transition, and virulence in pathogenic bacteria (Jenal et al. 2017; Römling and Simm 2009; Römling et al. 2005; Valentini and Filloux 2019). The synthesis and degradation of c-di-GMP are mediated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively. DGCs usually contain a conserved GGDEF domain, while PDEs typically have either an EAL domain or a HD-GYP domain (Bordeleau et al. 2011; Schirmer and Jenal 2009). Bacterial genomes usually encode a multitude of GGDEF, EAL, or HD-GYP domain proteins, many of which have been found in the genomes of diverse pathogenic bacteria (Römling et al. 2013). Cyclic di-GMP is sensed by a variety of receptors and effectors that control diverse cellular functions (Chou and Galperin 2016; Hengge 2009; Römling et al. 2013). Although many receptors or effectors, such as riboswitches, transcriptional regulators, PilZ domain proteins, GGDEF or EAL domain proteins, polynucleotide phosphorylases (PNPases), and kinases, have been shown to bind to c-di-GMP, it is likely that more receptors remain to be identified in bacteria (Chou and Galperin 2016).

Structural characterization and biological analysis have revealed that the RXXD motif of the I site in the GGDEF domain and the EXLXR motif in the EAL domain are crucial for c-di-GMP binding (Chou and Galperin 2016). Previous studies have indicated that degenerated GGDEF or EAL domain proteins without DGC or PDE activity, such as the

histidine protein kinase SgmT in *Myxococcus xanthus*, with a degenerated GGDEF domain, and LapD in *Pseudomonas fluorescens*, with a degenerated EAL domain, serve as c-di-GMP receptors (Newell et al. 2009; Petters et al. 2012). In addition, the *Xanthomonas* FimX protein, which possesses degenerated GGDEF and EAL domains, has been shown to bind c-di-GMP specifically via its EAL domain (Guzzo et al. 2009; Navarro et al. 2009). In *Xanthomonas axonopodis* pv. *citri*, FimX_{xac} interacts with the degenerated PilZ domain protein PilZ to regulate the bacterial type IV pilus biogenesis (Guzzo et al. 2009; Guzzo et al. 2013). This regulatory effect of FimX_{xac} and PilZ on type IV pilus biogenesis is mediated mainly through the formation of a complex with the ATPase PilB, which is required for type IV pilus polymerization (Guzzo et al. 2009, 2013). Filp, which is a FimX homolog protein in *X. oryzae* pv. *oryzae*, has been shown to control not only sliding motility but also bacterial virulence by interacting with PilZX3 (PXO_02715) (Yang et al. 2014). These studies suggested that the FimX homolog proteins and its interactors may function via a common but difference regulatory pathway in *Xanthomonas* spp. In addition to the degenerated GGDEF and EAL domain proteins, some DGCs or PDEs perform diverse functions by converting between the active enzyme form and a receptor. Whitney et al. (2012) and Chan et al. (2004) reported that the DGC activity of PleD and WspR is allosterically inhibited by the binding of c-di-GMP via the I site of the GGDEF domain. In *Escherichia coli*, two trigger PDEs, PdeR and PdeL, have been identified as c-di-GMP-sensing effectors. PdeR (YciR) inhibits the expression of biofilm regulator CsgD by interacting with the DGC DgcM and the transcription factor MlrA at low c-di-GMP levels. Interestingly, PdeR binds and degrades the c-di-GMP signal molecule at high c-di-GMP levels, thereby releasing DgcM and MlrA to activate the expression of the *csg* *DEFG* operon (Hengge 2016).

Xanthomonas oryzae pv. *oryzae* is the causative pathogen of bacterial leaf blight in rice (Niño-Liu et al. 2006). Diverse virulence factors such as extracellular polysaccharide (EPS), extracellular cellulases and xylanases, yellow pigments and adhesion molecules, the type II secretion system (T2SS), T3SS and its effectors, contribute to the pathogenicity of *X. oryzae* pv. *oryzae* in rice (Das et al. 2009; Kim et al. 2009; Rai et al. 2012). The expression of diverse virulence factors in *X. oryzae* pv. *oryzae* is controlled by c-di-GMP signaling (Yang et al. 2019). To date, several proteins involved in the production and sensing of c-di-GMP have been identified to regulate the virulence of *X. oryzae* pv. *oryzae*. Some of these members, including the GGDEF domain-containing DGCs GdpX1 and DgcA, the GGDEF and EAL domain-containing phosphodiesterase PdeR, the EAL domain-containing phosphodiesterase EdpX1, and the HD-GYP domain-containing phosphodiesterase RpfG, control the production of EPS, motility, biofilm formation, T3SS, and virulence of *X. oryzae* pv. *oryzae* by regulating the c-di-GMP concentration or through interactions with specific proteins (He et al. 2010; Su et al. 2016; Xue et al. 2018; Yang et al. 2012, 2016). The transcriptional regulator Clp, the degenerated GGDEF/EAL domain protein Filp, and the PilZ domain proteins PilZX1 (PXO_00049) and PilZX2 (PXO_02374) were shown to function as c-di-GMP receptors participating in regulation of virulence in *X. oryzae* pv. *oryzae* (Guan et al. 2009; Yang et al. 2014, 2015).

In this study, we investigated the mechanism by which Filp and PilZX3 coregulate virulence in *X. oryzae* pv. *oryzae* strains through relative and absolute quantitation (iTRAQ)-based quantitative proteomic analysis. We identified 157 DEPs as putative components regulated by Filp and PilZX3. The expression of selected DEPs was determined at the transcriptional and translational levels. The functions of selected DEPs in bacterial virulence were assessed by biochemical and genetic analyses. The results suggested a broader

regulatory influence of Filp and PilZX3 and implicated some of the regulatory components mediated by Filp and PilZX3 as novel virulence factors for *X. oryzae* pv. *oryzae*.

RESULTS

Gene deletion analysis revealed coregulatory roles of *filp* and *pilZX3* in virulence on rice.

Previous studies have shown that the c-di-GMP receptor Filp and the interactor PilZX3 protein are required for the full virulence of *X. oryzae* pv. *oryzae* through single mutation analysis (Yang et al. 2014). To further elucidate the mechanism by which *filp* and *pilZX3* regulate virulence, the double mutant $\Delta filp \Delta pilZX3$ was constructed. The PXO99^A cells and derived mutants were inoculated onto susceptible rice (*Oryza sativa* L. cv. Nipponbare) plant leaves by the leaf-clipping method. The disease symptoms were recorded by photography, and the lesion lengths were measured at 14 days postinoculation. The $\Delta filp \Delta pilZX3$ double mutant caused less severe disease symptoms (Supplementary Fig. S1A) and shorter lesion lengths (Supplementary Fig. S1B) compared with the wild-type strain ($P < 0.05$). These effects were similar to those caused by the *filp* or *pilZX3* single mutants (Supplementary Fig. S1A and B), which supports that idea that Filp and PilZX3 coregulate the virulence of *X. oryzae* pv. *oryzae* (Yang et al. 2014).

Filp/PilZX3-regulon revealed by iTRAQ-based proteomic analysis.

To identify novel proteins with a putative function in the virulence regulatory network mediated by Filp and PilZX3, we performed a quantitative iTRAQ-liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics analysis on the wild-type PXO99^A and *filp* or *pilZX3* single and double mutants. For the proteomics analysis, three independent cultures of the wild-type control and mutant strains were prepared in M210 nutrient-rich medium, and cells were collected in the exponential growth phase. Proteins were extracted and were subjected to the iTRAQ-LC-MS/MS proteomic analysis. Three independent biological replicate experiments were performed to account for biological variation. Using a peptide false discovery rate (FDR) ≤ 0.01 , we identified a total of 2,346 proteins representing approximately 51.2% of the 4,587 predicted proteins encoded by the *X. oryzae* pv. *oryzae* PXO99^A genome from a total of 28,403 peptides. The raw MS proteomics data have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository under the dataset identifier PXD015734.

Filp and PilZX3-mediated putative regulatory networks.

Using a cutoff of a 1.5-fold change in expression and a P value < 0.05 , we identified 283 DEPs (134 upregulated and 149 downregulated) in $\Delta filp$, 235 DEPs (90 upregulated and 146 downregulated) in $\Delta pilZX3$, and 222 DEPs (84 upregulated and 138 downregulated) in $\Delta filp \Delta pilZX3$ compared with the wild-type PXO99^A (Fig. 1; Supplementary File S1). In total, 157 DEPs (55 upregulated DEPs and 102 downregulated) were identified in the $\Delta filp$, $\Delta pilZX3$, and $\Delta filp \Delta pilZX3$ mutants (Supplementary File S1). In addition, 101 DEPs were observed only in the $\Delta filp$ mutant, 25 DEPs were observed only in the *pilZX3* mutant, and 14 DEPs were observed only in the *filp/pilZX3* double mutant (Fig. 1).

A total of 157 DEPs coregulated by Filp and PilZX3 were categorized into different functional groups of biological process, cellular component, and molecular function using gene ontology (GO) annotations in the UniProtKB database. The molecular functions of 157 DEPs were displayed mainly in catalytic activity (27%) and binding function (20%) categories

(Fig. 2A). In the biological process category, DEPs involved in metabolic progress (31%) and cellular progress (26%) were the most highly represented, although other biological functions such as establishment of localization and pigmentation, biological regulation, and localization were also represented (Fig. 2A). Cells and cell parts accounted for 17.2% of the DEPs in the cellular component category (Fig. 2A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the DEPs regulated by Filp and PilZX3 were involved in different signaling pathways, including metabolic pathways, biosynthesis of secondary metabolites, bacterial secretion systems, oxidative phosphorylation, bacterial chemotaxis, and two-component systems (TCS) (Fig. 2B). Among these, the histidine kinase (HK)-response regulator (RR) hybrid protein (HrrP) B2SMT1 in TCS, the type IV pilus-regulated proteins B2SVI4 and B2SVI3, and the TonB-dependent receptor B2SSR1 were significantly upregulated in all three mutants, while chemotaxis proteins B2SRN6 (designated EdpX2), B2SRN7 (designated CheW1) and B2SRN8, putative PDEs B2SW52 (designated HGdpX2) and B2SRN6, TonB-dependent receptor B2SWG3 (designated TdrX2), and HKs and RRs involved in TCS (B2SKU2, B2SRX4, B2SW53, HGdpX2, EdpX2) were significantly downregulated (Supplementary Fig. S3). Moreover, 17 proteins with varied expression levels in the $\Delta filp$, $\Delta pilZX3$, and $\Delta filp\Delta pilZX3$ mutants were implicated in bacterial T6SS, such as the type VI secretion ATPase (B2SI83, *clpV*) and the type VI protein secretion system components B2SRJ2 (*imcF*), B2SI89 (*evpB*), and B2SI85 (*evpF*).

In bacteria, diverse proteins can form complex networks with many interactions in different pathways. To better understand the regulatory mechanisms of *filp* and *pilZX3*, the potential protein network connections for DEPs regulated by Filp and PilZX were constructed using STRING. In total, 102 of the 157 DEPs of *filp* and *pilZX3* were mapped, mainly to two functional networks (group I and group II) (Fig. 2C). A specific network in group I had at least eight “focus” DEPs surrounding *filp* or *pilZX3*: B2SVI4 (*pilYI*), B2SVP3 (*fimV*), B2SRN6 (*PXO_04753*, designated as EdpX2), B2SW52 (*PXO_00476*, designated as HGdpX2), B2SKU2 (*PXO_02305*), B2SW53 (*PXO_00475*), B2SRN7 (*PXO_04752*, designated as CheW1), and B2SRN8 (*PXO_04751*) (Fig. 2C). The networks of interest corresponded to c-di-GMP signal degradation, TCS, chemotaxis proteins and type IV pilus regulation. The DEPs implicated in bacterial T6SSs and some of the uncharacterized proteins formed a complex interactive network in group II (Fig. 2C).

Fig. 1. Venn diagram showing the regulatory influence of *filp* and *pilZX3*. Data represent the number of proteins regulated by Filp or PilZX3. Numbers above and below lines indicate proteins that were upregulated and downregulated in *filp*, $\Delta pilZX3$, or $\Delta filp\Delta pilZX3$ mutants, respectively.

Alteration in abundance of Filp/PilZX3-regulated proteins is associated with transcription.

To validate the reliability of iTRAQ data, seven candidate DEPs (HrrP, PhrP, ProP, CheW1, EdpX2, HGdpX2, and TdrX2) that represented different molecular functions were selected for further validation by Western blot analysis (Supplementary Fig. S3). As shown in Figure 3A and B, the expression level of PhrP was significantly increased in the $\Delta pilZX3$ and $\Delta filp\Delta pilZX3$ mutants but not in the $\Delta filp$ mutant compared with that in PXO99^A. The expression of HrrP and ProP was significantly increased in mutants compared with that in the wild type, while the expression of CheW1, HGdpX2, and EdpX2 was decreased ($P < 0.05$) (Fig. 3A and B). Except for the expression of PhrP in the $\Delta filp$ mutant, the up-expression or down-expression trend of selected proteins were very similar to those shown by iTRAQ analyses (Fig. 3A and B), suggesting that the iTRAQ data were mostly reliable.

The altered abundance of proteins could be due to changes in transcriptional, translational, or post-translational regulation. The transcriptional levels of the DEPs were analyzed by quantitative reverse transcription-PCR (qRT-PCR) to determine their correlation with the changes in protein expression levels. Compared with the wild type, the mRNA levels of *hrrp*, *phrP*, and *proP* were significantly increased in the $\Delta filp$, $\Delta pilZX3$, and $\Delta filp\Delta pilZX3$ mutants, while the mRNA levels of *cheW1*, *edpX2*, *hGdpX2*, and *tdrX2* were significantly reduced ($P < 0.05$) (Fig. 3B). These observations suggested that Filp and PilZX3 negatively regulate the expression of Hrrp, PhrP, and ProP and positively regulate the expression of CheW1, EdpX2, HGdpX2, and TdrX2 at both the transcriptional and translational levels.

Novel components involved in Filp/PilZX3 regulation of virulence.

To investigate the function of regulatory components mediated by Filp and PilZX3 on bacterial virulence in *X. oryzae* pv. *oryzae*, we constructed the deletion mutants and complemented strains for *hrrp*, *phrP*, *proP*, *cheW1*, *edpX2*, *hGdpX2*, and *tdrX2*. The effects of these genes on *X. oryzae* pv. *oryzae* virulence were evaluated by inoculating susceptible rice plants (*Oryza sativa* subsp. *japonica*) with wild-type PXO99^A, mutant, and complemented strains. The disease symptoms were scored 14 days postinoculation. Compared with the wild-type strain, $\Delta cheW1$, $\Delta hGdpX2$, and $\Delta tdrX2$ caused less severe disease symptoms (Fig. 4A) and shorter lesion lengths (Fig. 4B). In addition, $\Delta hrrp$ and $\Delta proP$ caused more severe disease symptoms and longer lesion lengths, while $\Delta edpX2$ and $\Delta phrP$ did not obviously change the virulence of *X. oryzae* pv. *oryzae* (Fig. 4A and B). Complemented expression of the full-length genes in mutants resulted in almost complete restoration of the diseased phenotypes to that of the wild-type (Fig. 4A and B). This analysis revealed that the *cheW1*, *hrrp*, *hGdpX2*, *proP*, and *tdrX2* genes identified by comparative proteomics analysis contributed to the regulation virulence in *X. oryzae* pv. *oryzae*.

The regulatory components of Filp/PilZX3 influence the expression of virulence factors.

Our results showed that the selected DEPs regulated by Filp/PilZX3 contributed to the virulence of *X. oryzae* pv. *oryzae*; therefore, we investigated their influence on other virulence-related phenotypes, such as EPS production, motility, and biofilm formation. As shown in Supplementary Figure S4A, there was no significant difference between the wild type and mutants in terms of EPS secretion. Compared with wild-type PXO99^A, the biofilm formation of $\Delta cheW1$, $\Delta edpX2$, and $\Delta tdrX2$ mutants increased by approximately 55, 38, and 22%, respectively. In contrast, there were no significant differences in

biofilm formation between the $\Delta hGdpX2$, $\Delta hrrP$, $\Delta phrP$, and $\Delta proP$ mutants and wild-type PXO99^A (Supplementary Fig. S4B). Furthermore, $\Delta proP$ displayed increased sliding zones on SB media plates containing 0.6% agar while $\Delta edpX2$ displayed

decreased sliding zones, indicating that *proP* and *edpX2* regulate sliding motility of *X. oryzae* pv. *oryzae* (Supplementary Fig. S4C). In addition, the $\Delta hrrP$ strain showed a larger swimming zone than the wild-type PXO99^A on semisolid

Fig. 2. The classification of differentially expressed proteins (DEPs) regulated by Filp and PilZX3. **A**, Gene ontology analyses of DEPs. **B**, Kyoto Encyclopedia of Genes and Genomes classification of DEPs. **C**, Specific network analysis of proteins coregulated by Filp and PilZX3 by STRING analysis. Each node represents a protein. Colored nodes indicate query proteins and the first shell of the interactor; white nodes indicate the second shell of the interactor. Filled nodes represent known or predicted three-dimensional structures. Lines represent the interaction between proteins. Green-blue (from the curated database) and pink (experimentally determined) lines indicate known interactions; green (gene neighborhood), red (gene fusions), and blue (gene co-occurrence) lines indicate predicted interactions; yellow (text mining), black (coexpression), and blue (protein homolog) lines represent other interactions. The width of the line indicates the proximity of the relationship.

media plates containing 0.25% agar, whereas $\Delta cheW1$ and $\Delta hGdpX2$ showed smaller swimming diameters (Supplementary Fig. S4D). These observations suggested that these targets of Filp and PilZ3 were also involved in the regulation of biofilm formation and motility but not EPS production of *X. oryzae* pv. *oryzae*.

Both HGdpX2 and EdpX2 are PDEs active in c-di-GMP degradation.

SMART program analysis indicated that HGdpX2 contains a HD-GYP domain and EdpX2 harbors an EAL domain (Supplementary Fig. S3), which are responsible for c-di-GMP degradation (Hengge 2009). To evaluate potential PDE activity of HGdpX2 and EdpX2, we first compared the intracellular c-di-GMP levels in wild-type PXO99^A and mutants by LC-MS/MS. The results showed that the c-di-GMP concentration in *hGdpX2* and *edpX2* mutants was increased by approximately 24 and 25% of the wild-type level, respectively, while the wild-type levels were restored in the complementary strains (Fig. 5A). Next, we tested the PDE activity of HGdpX2 and EdpX2 in vitro. In colorimetric assays, incubation of SUMO-His₆-HGdpX2 and SUMO-His₆-EdpX2 with bis (*p*-nitrophenyl) phosphate overnight resulted in production of a significant amount of *p*-nitrophenol compared with that detected in the bovine serum albumin (BSA) control (Fig. 5B). Approximately 45 and 27% c-di-GMP were degraded by SUMO-His₆-HGdpX2 and SUMO-His₆-EdpX2, respectively, in the specified conditions, while no c-di-GMP degradation was observed in the BSA control (Fig. 5C). These results implied that HGdpX2 and EdpX2 were active PDEs in *X. oryzae* pv. *oryzae*.

DISCUSSION

C-di-GMP exerts diverse cellular functions at the transcriptional, translational, and post-translational levels (Chou and Galperin 2016; Hengge 2009; Römmling et al. 2013). Previous studies have revealed that homologs of FimX and its interacting PilZ domain protein in *Xanthomonas* spp. participate in type IV pilus assembly and regulation of bacterial virulence (Guzzo et al. 2009, 2013; Yang et al. 2014). Although protein interaction analysis has provided insights into the regulatory influence of FimXac and PilZ on type IV pilus, little is known about the mechanism by which Filp and PilZ3 regulate virulence. In this study, we showed that mutation of *filp* and *pilZ3* has a substantial effect on the *X. oryzae* pv. *oryzae* proteome, coaffecting the expression of 157 DEPs, including signal-sensing HKs and RRs in a TCS as well as PDEs and DGCs, chemotaxis proteins, TonB-dependent receptors, prophage proteins, and proteins involved in type IV pilus assembly or T6SSs. Thus, our findings provide further evidence that *filp* and *pilZ3* are functionally related. Among these DEPs, HGdpX2, CheW1 and TdrX2 positively regulated the virulence of *X. oryzae* pv. *oryzae*, while HrrP and ProP negatively regulated virulence (Fig. 3A), suggesting that these proteins are novel virulence regulators of *X. oryzae* pv. *oryzae* and that they are mediated by Filp and PilZ3.

Proteins involved in TCS, including two PDEs, were promoted by Filp and PilZ3.

It has been generally recognized that TCS composed of a signal-sensing HK and a RR with a receiver domain regulate gene expression or bacterial cellular behavior under different

Fig. 3. Expression of selected differentially expressed proteins (DEPs) determined in various *Xanthomonas oryzae* pv. *oryzae* strains by Western blot and quantitative PCR analyses. **A**, Western blot analyses comparing expression of DEPs in wild-type PXO99^A, $\Delta filp$, $\Delta pilZ3$, and $\Delta filp\Delta pilZ3$ mutants. Samples containing 10 μ g of protein were loaded in each lane and were analyzed by immunoblotting with corresponding antibodies. Protein expression of the housekeeping gene *GAPDH* was used as a reference. All individual blots were repeated three times using samples from independent cultures. **B**, The black columns represent the expression levels of DEPs quantified by isobaric tagging for relative and absolute quantitation. The white columns represent the expression levels of DEPs determined by Western blot analysis and quantified using Image J software. The grayscale values of bands were quantified to calculate the relative ratios of DEPs. The relative grayscale value in the wild type was arbitrarily designated as 1 for each reaction. The gray columns represent the expression levels of DEP-encoding genes were detected by quantitative reverse transcription PCR analysis using the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene *gyrB* was used as a reference. The expression level of each gene in the wild type was arbitrarily designated as 1 for each reaction. The experiment was repeated three times with similar results. 1 = PXO99^A, 2 = $\Delta filp$, 3 = $\Delta pilZ3$, 4 = $\Delta filp\Delta pilZ3$. One asterisk (*) indicates $P < 0.05$, two (**) indicates $P < 0.01$ by *t* test.

environmental conditions (Kulasekara et al. 2013). A large number of TCSs have been identified in the genome of *Xanthomonas campestris* pv. *campestris* (Qian et al. 2008). Although several proteins encoded by the *X. oryzae* pv. *oryzae* genome have been characterized as putative HKs or RRs, only a few have been experimentally confirmed (Lee et al. 2008).

Some of these proteins were found to be related to c-di-GMP metabolism, including the PDE PdeR with its cognate HK PdeK, the RR TriP and its PdeR interactor, and the HK RpfC with the PDE RpfG (He et al. 2010; Li et al. 2019; Yang et al. 2012, 2019). In this study, we showed that the expression of 11 putative components of TCS were regulated by Filp or PilZ_{X3}.

Fig. 4. Virulence test of mutant and complemented *Xanthomonas oryzae* pv. *oryzae* strains in rice. **A**, Rice plants were inoculated with *X. oryzae* pv. *oryzae* strains using the leaf-clipping method. The disease symptoms were observed 14 days after inoculation. **B**, Diseased lesion lengths were recorded. Data represent the mean $\pm$ standard deviation of at least 10 leaves. Three independent experiments were performed with similar results. An asterisk (*) indicates $P < 0.05$ by t test.

Among them, three of six HKs or RRs that were coregulated by Filp and PilZX3 were identified in this study. The putative HK-RR HrrP, which contains only a GAF signal-sensing domain, was shown to negatively regulate the virulence of *X. oryzae* pv. *oryzae*. Interestingly, the RR HGdpX2 containing a HD-GYP domain and EdpX2 containing an EAL domain were identified as PDEs that promote bacterial virulence or biofilm formation by modulating the intracellular c-di-GMP concentration in *X. oryzae* pv. *oryzae*. Intracellular c-di-GMP concentrations in bacteria are controlled by DGCs and PDEs (Schirmer and Jenal 2009). Expression of HGdpX2 and EdpX2 was positively regulated by Filp and PilZX3, indicating the possible existence of a feedback mechanism of c-di-GMP regulated by Filp and PilZX3. Moreover, PcrR, the homolog of HGdpX2 from *X. campestris* pv. *campestris*, formed a novel TCS with its cognate HK PcrK (the homolog of PXO_00475) and was involved in virulence regulation by degrading c-di-GMP (Wang et al. 2017). Interestingly, we also found that HK PXO_00475, which is the homolog of PcrK from *X. campestris* pv. *campestris*, was positively regulated by Filp and PilZX3. The interaction network analysis revealed that PXO_00475 was in the center of the interaction network of group II, suggesting PXO_00475 might be an important component controlled by Filp and PilZX3.

Proteins involved in the chemotaxis signaling pathway were upregulated by Filp and PilZX3.

The chemotaxis signaling pathway, which is a well-characterized model of signal transduction, participates in the regulation of the bacterial swimming/twitching motility, biofilm formation, and virulence (Matilla and Krell 2018). The main molecular components of the chemotaxis signaling transduction pathway are highly conserved in bacteria, including transmembrane

chemoreceptors, the cytoplasmic kinase CheA, the adaptor protein CheW, the RR CheY, the methyltransferase CheR, and the methyl-esterase CheB (Wuichet and Zhulin 2010). The chemotaxis signaling pathway in *X. oryzae* pv. *oryzae* is remarkably complex, with at least four chemotaxis (*che*) gene clusters encoding the components (Kumar Verma et al. 2018). In this study, 11 putative components of the chemotaxis signaling transduction pathway were found to be regulated by Filp or PilZX3 (Supplementary Fig. S1). Among them, CheW1(B2SRN4), EdpX2(B2SRN6, VieA), CheB1 (B2SRN4), and MCP (B2SRN8) in the *che1* gene cluster were coregulated by Filp and PilZX3. These results indicated a close connection between the chemotaxis signaling transduction pathway and Filp/PilZX3. Determination of the components of the chemotaxis signaling pathway in the *X. oryzae* pv. *oryzae* MAFF 311018 strain revealed that CheW1(XOO1468) positively regulates virulence, biofilm formation, and motility, while VieA (XOO1467) negatively regulates biofilm formation but positively regulates virulence (Kumar Verma et al. 2018). Deletion of CheB1 (XOO1469) decreased the virulence of *X. oryzae* pv. *oryzae* (Kumar Verma et al. 2018). CheW1, VieA, and CheB1 were required for the flagellar-mediated chemotactic response (Kumar Verma et al. 2018). In this study, we showed that CheW1 and EdpX2, which is the homolog of VieA in *X. oryzae* pv. *oryzae* MAFF 311018, were involved in the regulation of biofilm formation, motility, and virulence of the *X. oryzae* pv. *oryzae* PXO99^A strain. The regulatory effects of CheB1 and CheW1 on virulence were similar to those of Filp and PilZX3, which further supported the concept that the regulatory effects of these components are mediated via a similar pathway.

The novel virulence factors of *X. oryzae* pv. *oryzae* were regulated by Filp and PilZX3.

TonB-dependent receptors are outer membrane proteins that play important roles in the recognition of siderophores during iron uptake in gram-negative bacteria (Noinaj et al. 2010). Previous studies have revealed that some TonB-dependent receptors are involved in the regulation of bacterial virulence in *Xanthomonas* spp. For example, the TonB-dependent receptors XC4223, IroN, BfeA, and ButB are required for full virulence of *X. campestris* pv. *campestris*, while XCC3358 not only regulates the virulence of *X. campestris* pv. *campestris* in *Arabidopsis* but, also, the utilization of plant sucrose (Blanvillain et al. 2007; Qian et al. 2005). In *X. oryzae* pv. *oryzae*, TonB-dependent receptor TdrX1, which is the interactor of RR GacA, controls the bacterial growth, extracellular enzyme activity, motility, and virulence (Xu et al. 2010). Our data showed 11 TonB-dependent receptor/oar-like proteins were regulated by Filp or PilZX3, two of which were positively regulated. In this study, we also demonstrated that TdrX2 positively regulated virulence and negatively regulated biofilm formation. Therefore, we speculate that Filp and PilZX3 enhance nutrient uptake through upregulation of TonB-dependent receptors, thereby contributing to the virulence of *X. oryzae* pv. *oryzae*.

We also identified two components regulated by Filp and PilZX3, the PhoPQ-regulated protein PhrP and the prophage protein ProP, which negatively regulated the virulence and sliding motility of *X. oryzae* pv. *oryzae*. The TCS PhoPQ controls many pathogenic properties in several mammalian and plant pathogens (Moreira et al. 2015; Nakka et al. 2010). It is required for AvrXA21 activity, *hrpG* expression and virulence of *X. oryzae* pv. *oryzae* (Lee et al. 2008). The mild phage integrates its DNA into the host genome to become prophages, with approximately 65% of bacterial genomes carrying prophage sequences. Prophage proteins play an important role in bacterial toxin synthesis and secretion, genome variation and evolution, environmental adaptability, and represent many bacterial virulence factors (Fortier and Sekulovic 2013; Varani

Fig. 5. Both HGdpX2 and EdpX2 function as phosphodiesterases (PDEs) in vivo and in vitro. **A**, Intracellular c-di-GMP was extracted from *Xanthomonas oryzae* pv. *oryzae* wild type, mutants, and complement strains and was measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). **B**, PDE activity of HGdpX2 and EdpX2 was tested by in vitro colorimetric assay. Purified recombinant EdpX2-His6 and HGdpX2-His6 proteins were incubated with 5 mM bis-pNPP overnight at 37°C. Degradation of bis-pNPP into pNP was detected at 410 nm by spectrophotometry. **C**, PDE activity of HGdpX2 and EdpX2 was analyzed by LC-MS/MS. Purified proteins were incubated overnight at 37°C with 100 mmol c-di-GMP. The products were analyzed by LC-MS/MS. An asterisk (*) indicates $P < 0.05$ by t test.

et al. 2013; Zou et al. 2010). Interestingly, prophages contain riboswitches that are predicted to recognize c-di-GMP and are involved in multicellular behavior in *Clostridium difficile* (Fortier 2017). These findings suggested that these pathways are related to the c-di-GMP pathway that is regulated by Filp and PilZX3.

Two important bacterial secretion systems might be influenced by Filp and PilZX3.

Type IV pili, which are a widespread filamentous bacterial organelle, facilitate bacterial movement, adhesion, orientation, and multicellular organization (Mattick 2002). The extension, tethering, retraction, and extension of type IV pili is necessary for bacterial twitching/sliding motility. This organelle has garnered much attention in *Xanthomonas* spp. because type IV pili participate in pathogenesis, sliding/twitching motility, biofilm formation, and interactions with bacteriophages (Dunger et al. 2016). Our previous studies revealed that Filp interacted with PilZX3 to negatively regulate the sliding motility of *X. oryzae* pv. *oryzae*. In the present study, iTRAQ analysis showed that six proteins related to type IV pili assembly were markedly regulated by Filp or pilZX3, indicating that Filp and PilZX3 mediate sliding motility by regulating type IV pilus expression. PilX and PilY1 have been shown to be involved in the regulation of multiple bacterial biofunctions, including twitching motility in *Xylella fastidiosa*, virulence in *Pseudomonas aeruginosa*, and endothelial cell signaling in *Neisseria meningitidis* (Hlaine et al. 2005; Li et al. 2007; Marko et al. 2018). Mutation of PilY1 reduced the virulence in *X. oryzae* pv. *oryzicola* BLS303 (Wang et al. 2007). The expression of PilY1 and PilX were inhibited by Filp and PilZX3, suggesting that PilX and PilY1 might be involved in downstream regulators of sliding motility and virulence in *X. oryzae* pv. *oryzae*. FimX (the homolog of Filp), PilZ protein XAC1133 (the homolog of PilZX3), and ATPase PilB form a complex to promote the type IV pilus formation and inhibit sliding motility in *X. axonopodis* pv. *citri* (Guzzo et al. 2009, 2013; Qi et al. 2012). PilB (B2SSM0, the homolog of XAC1133), was downregulated in both the *pilZX3* single mutant and the *filp/pilZX3* double mutant but not in the *filp* mutant, suggesting that PilB is regulated by *pilZX3* alone. In addition, Filp acts independently to influence regulators of type IV pilus assembly, including the type IV pilus RR PilG (B2SSU9), the regulatory protein PilH family (B2SNJ7), and the type IV fimbriae assembly protein B2SNM9. PilG and PilH were an unlinked set of *pil* genes that were not in the pilus-regulated gene clusters. PilG was implicated in pilus extension and the role of PilH in regulating pilus retraction has been identified in *Pseudomonas aeruginosa* (Barken et al. 2008; Darzins and Russell 1997; Mattick et al. 1996). However, the contribution of PilG and PilH to virulence in *Xanthomonas* spp. remains unclear. Further identification of the biological roles of the type IV pilus in the virulence of *X. oryzae* pv. *oryzae* will help us understand the pathways regulated by Filp and PilZX3.

In addition, several proteins related to the T6SS were found to be regulated by Filp and PilZX in our iTRAQ analysis. The T6SS, a contact-dependent protein-secreting device that was first identified in *Vibrio cholerae*, injects a variety of effectors into eukaryotic and prokaryotic cells, thus playing important roles in bacterial interactions and virulence (Filloux et al. 2008; Pukatzki et al. 2009). T6SS is widely distributed in 25% of gram-negative bacteria with a highly conserved structure, including the 13 core components, a bacteriophage tail-like structure, and an injection system (Bernal et al. 2018). The c-di-GMP signaling pathway has been shown to participate in T6SS biosynthesis in *Pseudomonas* spp. In *Pseudomonas fluorescens*, c-di-GMP binds specifically and with high affinity to

the ATPase ClpB2 that is one of the core components of the T6SS (Trampari et al. 2015). In *Pseudomonas aeruginosa*, deletion of *retS* (encoding a sensor kinase) or overexpression of *wspR* (encoding a DGC) both lead to high intracellular levels of c-di-GMP, thereby upregulating the expression of T6SS. Inversely, overexpression of a PDE gene, *PA2133*, results in low c-di-GMP concentrations and inhibits the expression of T6SS (Moscoso et al. 2011). Studies have indicated that the c-di-GMP receptor FleQ and RopN cooperate to regulate the secretion of Hcp1 in T6SS at the transcriptional and post-transcriptional levels in *Pseudomonas putida* (Wang et al. 2018). T6SS is widespread in *Xanthomonas* spp., and the contribution of T6SS to bacterial competition but not virulence has been elucidated in *X. oryzae* pv. *oryzicola* (Ceseti et al. 2019; Zhu et al. 2019). The expression of several proteins related to T6SS that were upregulated by Filp and PilZX3 suggested that the c-di-GMP signaling pathway regulated by Filp and PilZX3 positively affects the expression of T6SS in *X. oryzae* pv. *oryzae* to allow the pathogen to gain a niche advantage during infection of rice.

A summary of the proteomic regulatory network of Filp and PilZX3 has been proposed (Fig. 6). The c-di-GMP receptor Filp and PilZX3 form a complex to regulate the virulence and sliding motility of *X. oryzae* pv. *oryzae* by promoting or suppressing signaling pathways, including TCS, the degradation of c-di-GMP, the chemotaxis signaling pathway, type IV pilus assembly, and the T6SS. In particular, crucial proteins in these signaling pathways and novel regulators that are regulated by Filp and PilZX3 contribute to the virulence of *X. oryzae* pv. *oryzae* in rice (Fig. 6). Overall, this study provides new insights into the mechanisms by which Filp and PilZX3 regulate virulence and provides insights into c-di-GMP signaling in *X. oryzae* pv. *oryzae*. T3SS and its effectors are the main virulence factors of *X. oryzae* pv. *oryzae* (White and Yang 2009). We have previously revealed that deletion of *filp* or *pilZX3* in *X. oryzae* pv. *oryzae* led to a decrease in T3SS gene expression at the transcriptional level (Yang et al. 2014). However, the influences of Filp and PilZX3 on T3SS were not characterized at the translational level in this study due to use of nutrient-rich medium rather than the minimal medium that simulated in planta to induce T3SS gene expression (Furutanin et al. 2003). Further investigation of the mechanism by which Filp and PilZX3 regulate the virulence of *X. oryzae* pv. *oryzae* in planta will help reveal the roles of c-di-GMP signaling in the interaction between pathogens and plants.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* cells were cultured at 37C in Luria-Bertani (LB) medium (Hanahan 1983). The *X. oryzae* pv. *oryzae* wild-type strain PXO99^A, mutants, and complementary strains were cultured at 28C on peptone sucrose agar (PSA) medium or M210 liquid medium with appropriate antibiotics (Hopkins et al. 1992). Relevant antibiotics were used at the following concentrations: ampicillin, 100 g ml^{1}; kanamycin, 50 g ml^{1}; and gentamycin, 20 g ml^{1} (Winiewski et al. 2009).

Construction of mutants and complementation.

To generate in-frame gene deletions, the left and right fragments of *pilZX3*, *hrrP*, *phrP*, *proP*, *cheW1*, *edpX2*, *hGdpX2*, and *tdrX2* were amplified, using *X. oryzae* pv. *oryzae* genomic DNA as the template, with specific primers (Supplementary Table S1). The DNA fragments were inserted into the pKMS1 vector to generate plasmids pKMSpilZX3, pKMShrrP, pKMSphrP,

pKMSproP, pKMScheW1, pKMShGdpX2, and pKMStdrX2. The plasmids were used to delete target genes in the *X. oryzae* pv. *oryzae* genome through homologous recombination, as described previously (Zou et al. 2011). pKMSpilZX3 was electroporated into the *filp* mutant to generate the *filp/pilZX3* double mutant. The pKMShrrP, pKMSphrP, pKMSproP, pKMScheW1, pKMShGdpX2, and pKMStdrX2 constructs were introduced into PXO99^A by electroporation. The potential mutants were screened as described by Zou et al. (2011). Single colonies that grew on PSA plates containing 10% sucrose but were sensitive to kanamycin were selected to validate the mutants by PCR. To generate the complemented strains, the coding region of *hrrP*, *phrP*, *proP*, *cheW1*, *hGdpX2*, and *tdrX2*, including the ribosomal binding sites, were amplified using corresponding primer pairs (Supplementary Table S1). The PCR fragments were ligated into the pBBR1MCS-4 vector to generate the pBhrrP, pBphrP, pBproP, pBcheW1, pBhGdpX2, and pBtdrX2 constructs, which were electroporated into the corresponding mutant strains for complementation analysis.

iTRAQ analysis of the *X. oryzae* pv. *oryzae* proteome.

Protein preparation and digestion. Previous studies have revealed that there are no differences in the growth characteristics of wild-type PXO99^A and the *filp* and *pilZX3* single mutants (Yang et al. 2014). The same results were observed for

the wild-type PXO99^A and the *filp* and *pilZX3* double mutant (data not shown). The wild-type PXO99^A, $\Delta filp$, $\Delta pilZX3$, and $\Delta filp\Delta pilZX3$ *X. oryzae* pv. *oryzae* strains were cultured to the exponential growth phase indicated by an optical density at 600 nm (OD₆₀₀) of 0.8. Briefly, *X. oryzae* pv. *oryzae* strains were cultured in M210 medium overnight at 28°C and were then transferred to 100 ml of fresh M210 medium. The concentrations of bacteria were adjusted to OD₆₀₀ = 0.05. These strains were then cultured at 28°C in a rotating incubator (200 rpm) for 12 to 24 h. The OD₆₀₀ of *X. oryzae* pv. *oryzae* strains was monitored until it reached 0.8. Three biological replicates were prepared for each strain. Bacterial cells were collected and were washed twice with sterilized phosphate buffered saline (PBS). The cells were resuspended with HEPES buffer (20 mM HEPES, 9 M urea, pH 8.0) and were mixed with quartz sand before bursts (10 times for 20 s) of vigorous agitation at maximum speed, with incubation on ice for 5 min between each step. The samples were then lysed by bursts of ultrasonic treatment (power 80 W, 10 times for 10 s, interval time 15 s) on ice. The supernatants were then collected by centrifugation at 13,400 rpm for 30 min and the protein concentration was evaluated by the Bradford method (Tiangen). Equal amounts of proteins from each sample were digested with trypsin at 37°C for 16 to 18 h, according to the method described by Wiśniewski et al. (2009), and finally, were lyophilized.

Fig. 6. A summary of the proteomic regulatory network of Filp and PilZX3 in *Xanthomonas oryzae* pv. *oryzae*. The degenerate GGDEF/EAL domain protein Filp binds to c-di-GMP through its EAL domain and interacts with the degenerate PilZ domain protein PilZX3 to regulate the sliding motility and virulence of *X. oryzae* pv. *oryzae* (Yang et al. 2014). Several important signaling pathways including two-component systems, degradation of c-di-GMP, the chemotaxis signaling pathway, type IV pilus assembly, and the type VI secretion system are regulated by Filp and PilZX3. The represented differentially expressed proteins (DEPs) CheW1, ProP, HrrP, and HGdpX2 contribute to the virulence of *X. oryzae* pv. *oryzae* in rice. Besides the DEPs-related type IV pili assembly, EdpX2, ProP, and TdrX2 involve the regulation of sliding motility of *X. oryzae* pv. *oryzae*. In addition, CheW1, HrrP, EdpX2, and TdrX2 also affect the biofilm formation or swimming motility. The ovals filled in red and blue indicate the upregulated and downregulated DEPs in the $\Delta filp$, $\Delta pilZX3$, and $\Delta filp\Delta pilZX3$ mutants, respectively. Arrowheads at the end of lines represent positive regulated trends and bars at the end of lines indicate negative regulated trends.

iTRAQ sample labeling and LC-MS/MS analysis. The protein labeling was carried out according to manufacturer instructions (AB Sciex). Four groups of proteins were labeled with the reporter tags 114 (PXO99^A), 116 (Δ *filP*), 115 (Δ *pilZX3*), and 113 (Δ *filP* Δ *pilZX3*), respectively. The iTRAQ-labeled peptides were separated by strong-cation exchange chromatography on the AKTA purifier system (GE Healthcare). The labeled peptides were fractionated and acidified before loading onto a polysulfoethyl column (5 μ m, 4.6 $\times$ 100 mm column; PolyLC Inc.). The collected fractions were combined into five pools and were then desalted on C18 cartridges. The fractions were vacuum-centrifuged to dryness and were dissolved in 0.1% trifluoroacetic acid.

(MS/MS analysis was performed on a Q-extracted mass spectrometer (Thermo Fisher Scientific). For each fraction, peptide mixtures were injected into a C18-reversed phase high-pressure liquid chromatography (HPLC) column (3 μ m, 7.5 $\times$ 100 mm; Thermo Scientific Easy Column), were separated at a flow rate of 250 nl/min and a linear gradient of acetonitrile in buffer B (80% acetonitrile and 0.1% formic acid) for 140 min. A data-dependent top-10 method was used to acquire the MS data and select the most abundant precursor ions in the range of *m/z* 300 to 1,800 from the survey scan for HCD fragmentation. The target value was determined based on predictive automatic gain control and the dynamic exclusion duration was 60 s. The parameters of the second level of MS were as follows: resolution of 17,500 at *m/z* 200, 30 eV of normalized collision energy, and 0.1% of the underfill rate.

Sequence database searching and data analysis. For quantification and identification of proteins, the MS/MS spectra were submitted to MASCOT (version 2.2; Matrix Science) using Proteome Discoverer 1.3 (Thermo Electron). The UniProt *Xanthomonas oryzae* pv. *oryzae* PXO99^A database (133,549 sequences, downloaded July 10, 2017) was used for the MS/MS data search. The FDR for peptides was set to 0.01 to filter the effective peptides. The proteins with a 1.5-fold change in expression and a *P* value <0.05 were considered as differentially expressed protein.

GO analyses were conducted using the GO database. The KEGG database was used to predict the metabolic pathway of the proteins. The functional associations between proteins were analyzed using the STRING database. A heatmap was created to show the expression level of proteins using the Pheatmap function in the R package (version 3.4.3) (Kolde 2015).

Protein expression, purification, and antibody preparation.

The DNA fragments encoding the *hrrP*, *phrP*, *proP*, *cheW1*, *edpX2*, *hGdpX2*, and *tdrX2* genes were amplified using corresponding primer pairs (Supplementary Table S1). The fragments were cloned into pColdSUMO to generate the pCHrrP, pCPhrP, pCProP, pCCheW1, pCEdpX2, pCHGdpX2, and pCTdrX2 plasmids and were verified by sequencing. The plasmids were transformed into *E. coli* BL21 strains and the

Table 1. The bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80(<i>lacZ</i>) Δ <i>lacX74</i> hsdR(γ_k , m_k^+) Δ <i>recA1398</i> endA1tonA	TransGene Biotech, Beijing
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Wild-type strain, Philippine race 6, Cp ^r	Our laboratory
Δ <i>filP</i>	<i>filP</i> gene deletion mutant derived from PXO99 ^A , Gm ^r , Cp ^r	Yang et al. 2014
Δ <i>pilZX3</i>	<i>pilZX3</i> gene deletion mutant derived from PXO99 ^A , Gm ^r , Cp ^r	Yang et al. 2014
Δ <i>filP</i> Δ <i>pilZX3</i>	<i>filP</i> and <i>pilZX3</i> gene double deletion mutant derived from PXO99 ^A , Gm ^r , Cp ^r	This study
Δ <i>hrrP</i>	<i>hrrP</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>phrP</i>	<i>phrP</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>proP</i>	<i>proP</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>cheW1</i>	<i>cheW1</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>edpX2</i>	<i>edpX2</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>hGdpX2</i>	<i>hGdpX2</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ <i>tdrX2</i>	<i>tdrX2</i> gene deletion mutant derived from PXO99 ^A , Cp ^r	This study
Plasmid		
pKMS1	Suicidal vector carrying <i>sacB</i> gene for mutagenesis, Km ^r	(Schäfer et al. 1994)
pBBR1MCS-4	Broad-host range expression vector, Ap ^r	(Hopkins et al. 1992)
pEASY-Blunt	Cloning vector, Ap ^r	TransGen Biotech
pColdSUMO	Expression vector to generate a N-terminal SUMO-His ₆ -tag, Ap ^r	Haigene
pKMSPilZX3	pKMS1 with <i>pilZX3</i> , Km ^r	This study
pCHrrP	pColdSUMO containing the <i>HrrP</i> coding sequence, Ap ^r	This study
pCPhrP	pColdSUMO containing the <i>PhrP</i> coding sequence, Ap ^r	This study
pCProP	pColdSUMO containing the <i>ProP</i> coding sequence, Ap ^r	This study
pCCheW1	pColdSUMO containing the <i>CheW1</i> coding sequence, Ap ^r	This study
pCEdpX2	pColdSUMO containing the <i>EdpX2</i> coding sequence, Ap ^r	This study
pCTdrX2	pColdSUMO containing the <i>TdrX2</i> coding sequence, Ap ^r	This study
pKMS _{hrrP}	pKMS1 with <i>hrrP</i> , Km ^r	This study
pKMS _{phrP}	pKMS1 with <i>phrP</i> , Km ^r	This study
pKMS _{proP}	pKMS1 with <i>proP</i> , Km ^r	This study
pKMS _{cheW1}	pKMS1 with <i>cheW1</i> , Km ^r	This study
pKMS _{edpX2}	pKMS1 with <i>edpX2</i> , Km ^r	This study
pKMS _{tdrX2}	pKMS1 with <i>tdrX2</i> , Km ^r	This study
pB _{hrrP}	pBBR1MCS-4 carrying the full-length of <i>hrrP</i> , Ap ^r	This study
pB _{phrP}	pBBR1MCS-4 carrying the full-length of <i>phrP</i> , Ap ^r	This study
pB _{proP}	pBBR1MCS-4 carrying the full-length of <i>proP</i> , Ap ^r	This study
pB _{cheW1}	pBBR1MCS-4 carrying the full-length of <i>cheW1</i> , Ap ^r	This study
pB _{edpX2}	pBBR1MCS-4 carrying the full-length of <i>edpX2</i> , Ap ^r	This study
pB _{hGdpX2}	pBBR1MCS-4 carrying the full-length of <i>tdrX2</i> , Ap ^r	This study
pB _{tdrX2}	pBBR1MCS-4 carrying the full-length of <i>tdrX2</i> , Ap ^r	This study

^a Km^r, Ap^r, Gm^r, and Cp^r indicate resistance to kanamycin, ampicillin, gentamicin, and chloramphenicol, respectively.

expression of the target proteins was induced by addition of 0.5 mM isopropyl- β -D-thiogalactoside, followed by incubation at 16°C for 12 h. The protein was purified according to previously described methods (Yang et al. 2012, 2014). New Zealand white rabbits were immunized with 6 mg of each recombinant protein to prepare the specific monoclonal antibodies (Wuhan GeneCreate Biological Co.), which were then identified by coimmunoprecipitation and Western blot analyses.

Western blotting analysis.

Bacterial strains were cultured in M210 medium at 28°C to OD₆₀₀ of 1.0. The cells were collected by centrifugation and were washed twice with sterilized PBS. The proteins were then extracted using the Minute Kit (Invent Biotech). Eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gels) and were transferred onto membranes for immunoblotting with corresponding primary antibodies that were generated in this study. Goat antirabbit secondary antibodies conjugated with horseradish peroxidase were used to recognize the primary antibodies. The results were visualized by addition of the diaminobenzidine substrate (Tiangen Biotech).

qRT-PCR analysis.

Bacterial cells were collected after culture in M210 at 28°C to an OD₆₀₀ of 0.8. Total RNA was extracted using the RNeasy pure cell/bacteria kit (Tiangen). qPCRs were conducted using AceQ qPCR SYBR Green Master Mix (Vazyme) according to manufacturer instructions. The *gyrB* gene was used as an endogenous control for data normalization. The relative expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Three biological replicates were tested for each sample, with three technical replicates in each experiment. Details of the gene-specific primers are listed in Supplementary Table S1.

Virulence test.

Virulence was assessed in pathogenicity assays on susceptible rice plants (*Oryza sativa* L. cv. Nipponbare) following inoculation using the leaf-clipping method (Yang et al. 2012). The *X. oryzae* pv. *oryzae* strains were grown in M210 media at 28°C to an OD₆₀₀ of 1.0. The bacterial cells were resuspended in an equal volume of sterile distilled water, then, were inoculated onto rice leaves. Photos were taken and lesion lengths were measured at 14 days after inoculation. The experiments were repeated at least three times.

Motility assay.

Fresh *X. oryzae* pv. *oryzae* colonies were stabbed into semisolid plates containing 0.03% agar (Shen et al. 2001), for evaluation of swimming motility, and SB plates containing 0.5% (wt/vol) Bacto peptone, 0.5% yeast extract, 0.1% L-glutamic acid, 0.5% sugar, pH 7.0, and 0.6% agar 0.6% agar (McCarthy et al. 2008), for evaluation of sliding motility. The plates were inoculated at 28°C for 4 days. The diameters of the swimming and sliding motility zones were measured in millimeters. This assay was repeated at least three times.

EPS production assay.

EPS production by *X. oryzae* pv. *oryzae* strains was quantified using the ethanol precipitation method (Tang et al. 1996). *X. oryzae* pv. *oryzae* strains were grown in M210 medium for 48 h. The culture supernatants were then mixed with two volumes of absolute ethanol and were stored overnight at 20°C. EPS molecules were collected by centrifugation at 12,000 $\times$ g for 20 min, were dried overnight at 55°C, and were weighed. The experiment was independently repeated three times.

Biofilm formation assay.

Bacteria in the logarithmic growth phase were diluted in M210 medium to an OD₆₀₀ of 0.5. Bacteria were incubated at 28°C for 4 days. Bacterial pellicles were stained with 0.1% crystal violet for 15 min at room temperature, then, were gently rinsed three times with distilled water. Biofilm formation was quantified by dissolving the adherent dye with ethanol and measuring the absorbance at 490 nm (An et al. 2010).

Quantification of the intracellular c-di-GMP levels in

X. oryzae pv. *oryzae* strains.

The c-di-GMP in bacteria was extracted and quantified as previously described (Xue et al. 2018). *X. oryzae* pv. *oryzae* strains were cultured at 28°C in 200 ml of M210 medium to OD₆₀₀ of 2.0. The bacterial cells were collected by centrifugation at 12,000 $\times$ g for 20 min and were resuspended in 1.5 ml of extraction buffer (40% methanol, 40% acetonitrile). The mixtures were sonicated for 10 min, following incubation on ice for 15 min. After heating for 10 min at 95°C, the suspension was collected by centrifugation at 13,000 $\times$ g for 20 min. The suspension was dried and dissolved with 100 μ l of HPLC-grade water. Then, 10 μ l of each sample was analyzed on an Agilent 1290 (Agilent) liquid chromatography system equipped with a QQQ (Agilent 6460) tandem mass spectrometer. Commercially synthesized c-di-GMP (Biolog) was used as a reference for quantification of c-di-GMP.

PDE activity analysis.

PDE activity of proteins was analyzed by colorimetric assay and LC-MS/MS analysis as previously described (Yang et al. 2012; Yi et al. 2010). For colorimetric assays, the reaction system containing 100 μ g of purified protein and 5 mM bis (*p*-nitrophenyl) phosphate in reaction buffer (50 mM Tris-HCl, 1 mM MnCl₂, pH 8.5) was incubated at 37°C for 2 h. The yellow product (*p*-nitrophenyl) was detected by measuring the OD₄₁₀ nm. In addition, 100 μ g of purified proteins were incubated with 10 μ M c-di-GMP in 1 ml of reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM MnCl₂, 0.5 mM EDTA, and 50 mM NaCl) overnight at 37°C. BSA was used as a negative control. The products were then measured by reverse phase-coupled LC-MS/MS analysis (Xue et al. 2018).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Gene Ontology database: <http://geneontology.org>
Kyoto Encyclopedia of Genes and Genomes (KEGG) database: <https://www.genome.jp/kegg>
Cran R Pheatmap function: cran.r-project.org/web/packages/pheatmap/index.html
The ProteomeXchange Consortium: <http://proteomecentral.proteomexchange.org>
STRING database: <https://string-db.org>
The UniProtKB database: <https://www.uniprot.org>

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