

# The *Xanthomonas oryzae* pv. *oryzae* PilZ Domain Proteins Function Differentially in Cyclic di-GMP Binding and Regulation of Virulence and Motility

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The PilZ domain proteins have been demonstrated to be one of the major types of receptors mediating cyclic di-GMP (c-di-GMP) signaling pathways in several pathogenic bacteria. However, little is known about the function of PilZ domain proteins in c-di-GMP regulation of virulence in the bacterial blight pathogen of rice *Xanthomonas oryzae* pv. *oryzae*. Here, the roles of PilZ domain proteins PXO\_00049 and PXO\_02374 in c-di-GMP binding, regulation of virulence and motility, and subcellular localization were characterized in comparison with PXO\_02715, identified previously as an interactor with the c-di-GMP receptor Filp to regulate virulence. The c-di-GMP binding motifs in the PilZ domains were conserved in PXO\_00049 and PXO\_02374 but were less well conserved in PXO\_02715. PXO\_00049 and PXO\_02374 but not PXO\_02715 proteins bound to c-di-GMP with high affinity *in vitro*, and the R<sup>141</sup> and R<sup>10</sup> residues in the PilZ domains of PXO\_00049 and PXO\_02374, respectively, were crucial for c-di-GMP binding. Gene deletion of PXO\_00049 and PXO\_02374 resulted in significant increases in virulence and *hrp* gene transcription, indicating their negative regulation of virulence via type III secretion system expression. All mutants showed significant changes in sliding motility but not exopolysaccharide production and biofilm formation. In *trans* expression of the full-length open reading frame (ORF) of each gene in the relevant mutants led to restoration of the phenotype to wild-type levels. Moreover, PXO\_00049 and PXO\_02374 displayed mainly multisite subcellular localizations, whereas PXO\_02715 showed non-polar distributions in the *X. oryzae* pv. *oryzae* cells. Therefore, this study demonstrated the different functions of the PilZ domain proteins in mediation of c-di-GMP regulation of virulence and motility in *X. oryzae* pv. *oryzae*.

Cyclic dimeric GMP (c-di-GMP) was originally identified as a positive allosteric regulator of cellulose synthase in *Gluconacetobacter xylinus* (1). Now, it has been recognized as an important intracellular signaling molecule implicated in the control of various bacterial properties, including motility, biofilm formation, adherence, and production of virulence factors (2, 3). The intracellular level of c-di-GMP is controlled by diguanylate cyclases (DGCs), which contain the GGDEF domain, and phosphodiesterases (PDEs), which harbor EAL or HD-GYP domains (4). Also, the downstream cellular functions regulated by c-di-GMP often depend on signal transduction by its receptors or effectors. A wide variety of c-di-GMP receptors have been discovered, such as PilZ domains (5), the transcription factors (6–8), proteins containing degenerate GGDEF or EAL domains (9–11), and the RNA processing polynucleotide phosphorylase (PNPase) and riboswitches (12–14). Therefore, c-di-GMP can exert a regulatory function at various levels of gene transcription, translation, and posttranslational.

The PilZ domain is the first c-di-GMP binding module identified in *G. xylinus* and is named PilZ domain after being identified from *Pseudomonas aeruginosa* (5). Two highly conserved motifs, RxxxR and N/DxxxxG, in the PilZ domain are recognized to bind c-di-GMP by the crystal structure of the PilZ domain–c-di-GMP complex and biological analysis (15). PilZ domains are widely distributed in bacterial genomes, and bacterial genomes generally encode one or multiple PilZ domain proteins which contain a single PilZ domain alone or conjugated with other domains. Many PilZ domain proteins have been experimentally identified in different bacteria and implicated in the regulation of motility, polysaccharide synthesis, biofilm formation, and virulence. YcgR from

*Escherichia coli* and *Salmonella* sp. was shown to regulate flagellar motility by directly interacting with flagellar motor components FliG and FliM in the presence of c-di-GMP (15–18). The PilZ domain proteins Alg44 and FlgZ in *P. aeruginosa* are required for alginate biosynthesis, swimming motility, and biofilm formation (19, 20). In *Vibrio cholerae*, five PilZ domain proteins function as c-di-GMP receptors, which are involved in the regulation of biofilm formation, motility, and virulence (21). Four PilZ domain proteins (XC\_0965, XC\_02249, XC\_2317, and XC\_3221) have been reported to be involved in the virulence regulation of *Xanthomonas campestris* pv. *campestris* (22). These observations suggest that downstream regulation mediated by PilZ domain proteins in the c-di-GMP signal pathway varies in different bacterial species.

*X. oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice,

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has been one of the model systems to study the molecular mechanism of bacterial pathogenesis in plants (23). *X. oryzae* pv. *oryzae* produces many virulence factors, such as exopolysaccharide (EPS), extracellular enzymes, adhesins, and the type III secretion system (T3SS) and its effectors (24). For the type III secretion system of *X. oryzae* pv. *oryzae*, HrpG and HrpX are two master regulators which control the expression of *hrp* genes and type III effectors. In the genome of *X. oryzae* pv. *oryzae* PXO99<sup>A</sup>, 26 genes encoding GGDEF, EAL, or HD-GYP domain proteins have been identified through bioinformatics analysis (25). Some of these proteins have been identified as PDEs to regulate EPS production and virulence in *X. oryzae* pv. *oryzae*, such as PdeR and RpfG, which were response regulators in two-component systems (25, 26). However, little is known about the downstream regulation of the c-di-GMP signal pathway in *X. oryzae* pv. *oryzae*. The transcription factor Clp and degenerate GGDEF and EAL domain protein Filp have been identified as c-di-GMP receptors in *X. oryzae* pv. *oryzae* (11, 27). Interestingly, Filp specially interacts with PilZ domain protein PXO\_02715 to regulate bacterial virulence (11). There are three PilZ-encoding sequences, *PXO\_00049*, *PXO\_02374*, and *PXO\_02715*, in the *X. oryzae* pv. *oryzae* PXO99<sup>A</sup> genome (28), which showed protein similarities of 85.4%, 85.5%, and 92.1%, respectively, to the PilZ domain proteins XC\_2317, XC\_0965, and XC\_3221 from *X. campestris* pv. *campestris*. Our previous studies have shown that *PXO\_02715* is required for the full virulence and T3SS-related *hrp* gene expression in *X. oryzae* pv. *oryzae* (11). However, whether other PilZ domain proteins besides *PXO\_02715* bind to c-di-GMP and participate in regulation of *X. oryzae* pv. *oryzae* virulence and motility remain unknown.

In this work, we identified these PilZ domain proteins and examined their c-di-GMP binding ability and function in regulating virulence and motility of *X. oryzae* pv. *oryzae*. Our evidence showed specific c-di-GMP binding *in vitro* of *PXO\_00049* and *PXO\_02374* but not *PXO\_02715*. The *PXO\_00049* gene deletion resulted in increased virulence and decreased sliding motility, and the *PXO\_02374* mutation caused increased virulence and sliding motility of *X. oryzae* pv. *oryzae*. Meanwhile, the expression of type III secretion system (T3SS)-related genes (*hrpX*, *hrpG*, and *hpa1*) was regulated by *PXO\_00049* and *PXO\_02374*. Moreover, subcellular localization analysis showed that the spatial distribution of three PilZ domain proteins varied in *X. oryzae* pv. *oryzae* cells. These results imply that PilZ domain proteins function differentially in c-di-GMP binding, regulation of bacterial virulence and motility, and subcellular localization in *X. oryzae* pv. *oryzae*.

## MATERIALS AND METHODS

All biological experiments with the pathogens described in this publication were conducted at the Chinese Academy of Agricultural Sciences in Beijing, China.

**Maintenance of bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium at 37°C (29). *X. oryzae* pv. *oryzae* strain PXO99<sup>A</sup> and derived mutant strains were cultured at 28°C on peptone sucrose agar (PSA) medium or M210 liquid medium with appropriate antibiotics (30). The antibiotics used were ampicillin (Ap), kanamycin (Km), and gentamicin (Gm) at concentrations of 100 µg ml<sup>-1</sup>, 50 µg ml<sup>-1</sup>, and 20 µg ml<sup>-1</sup>, respectively.

**Protein expression and purification.** The DNA fragments encoding *PXO\_00049* and *PXO\_02374* were amplified by primer pairs 49PF1/49PR1 and 2374PF1/2374PR1, respectively. For the mutation of R<sup>141</sup> of

*PXO\_00049*, the upstream and downstream fragments were amplified using primers 49PF1/49PR2 and 49PF2/49PR1. Then, two fragments were used as the templates to amplify fragment 49R<sup>141</sup>D by primers 49PF1/49PR1. We also amplified 2374R<sup>10</sup>D using the same methods. The primers are listed in Table S1 in the supplemental material. The PCR fragments were gel purified and cloned into the pMD18-T vector (TaKaRa, Tokyo, Japan), following verification by sequencing. The fragments were digested with the appropriate restriction enzymes and cloned into pColdSUMO, resulting in plasmids pC49, pC2374, pC49R<sup>141</sup>D, and pC2374R<sup>10</sup>D. The plasmids were transformed into *E. coli* BL21 strains for expression of the protein. Also, the *E. coli* strain used for the expression of *PXO\_02715* was from our previous study (11). Expression of the target protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM, and the bacterial cultures were collected after incubation at 16°C for 12 h. Subsequently purification of the protein was performed according to the methods described by Yang et al. (11, 25). SUMO-His<sub>6</sub> tag expressed from the empty vector pColdSUMO was purified as a negative control.

**Isothermal titration calorimetry (ITC) assay.** The binding analysis of the proteins was carried out on an ITC200 calorimeter (MicroCal, Northampton, MA), according to the manufacturer's directions. In brief, 2 µl c-di-GMP solution (200 µM, 300 µM, or 500 µM) was injected at 2-min intervals via a 60-µl syringe into the sample cell containing 30 µM SUMOHis<sub>6</sub>-*PXO\_00049*, 50 µM SUMOHis<sub>6</sub>-*PXO\_02374m*, 50 µM SUMOHis<sub>6</sub>-*PXO\_02715*, or 20 µM SUMOHis<sub>6</sub> tag protein with constant stirring, and the heat changes accompanying these additions were recorded. The titration experiment was repeated three times, and the data were calibrated with a buffer control and fitted with the single-site model to determine the dissociation constant (*K<sub>d</sub>*) using the MicroCal ORIGIN version 7.0 software.

**Construction of gene deletion mutant and complementation analysis.** For generating the mutants, homologous recombination as described previously was used in this study (31). The upstream and downstream fragments of *PXO\_00049* were amplified using chromosomal DNA of PXO99<sup>A</sup> as the template with 49MF1/49MR1 and 49MF2/49MR2 as primers. The DNA fragments corresponding to downstream and upstream regions of *PXO\_02374* were amplified using specific primers 2374MF1/2374MR1 and 2374MF2/2374MR2, respectively. The DNA fragment was digested with corresponding restriction enzymes and ligated to pK18mobsacB. A gentamicin resistance (Gm<sup>r</sup>) gene was amplified from pBBR1MCS-5 plasmid (32) using the primers GmF/GmR and digested with BamHI and HindIII, and then each gene fragment carried by pK18mobsacB was inserted, resulting in plasmids pK49G and pK2374G. pK49G and pK2374G were introduced into PXO99<sup>A</sup> by electroporation. Potential mutants were selected on PSA plates containing gentamicin and 10% sucrose. The single colonies that were resistant to gentamicin and high concentrations of sucrose but sensitive to kanamycin were further confirmed as mutants by sequencing the PCR products.

For complementation analysis, coding regions for the full length of *PXO\_00049* and *PXO\_02374* were amplified by primer pairs 49CF/49PR1 and 2374CF/2374PR1, respectively. *PXO\_00049*R<sup>141</sup>D and *PXO\_02374*R<sup>10</sup>D fragments were amplified using the primers 49CF/49PR1 and 2374CF/2374PR1 by pC49R<sup>141</sup>D and pC2374R<sup>10</sup>D as the template, respectively. The information on the primers is listed in Table S1 in the supplemental material. The PCR fragments were gel purified and cloned into the middle vector pMD18-T for sequencing, and each fragment was digested from pMD18-T-derived constructs with KpnI/HindIII and ligated into the vector pBBR1MCS-4 (32), resulting in constructs pB49, pB2374, pB49R<sup>141</sup>D, and pB2374R<sup>10</sup>D to express the proteins under the *lac* promoter. These constructs were electroporated into the Δ*PXO\_00049* or Δ*PXO\_02374* mutant for complementation studies. The *PXO\_02715* deletion mutant and complementation strain were from our previous work (11).

**Virulence test.** The wild-type and mutant strains of PXO99<sup>A</sup> were grown for 72 h at 28°C in M210 medium with appropriate antibiotics. The

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<b>Strains</b>		
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>supE44 <math>\Delta</math>lacU169(<math>\phi</math>80lacZ<math>\Delta</math>M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	27
BL21	For protein expression	Novagen
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 <sup>A</sup>	Wild-type strain, Philippine race 6	Lab collection
$\Delta$ PXO_00049 mutant	PXO_00049 gene deletion mutant derived from PXO99 <sup>A</sup> , Gm <sup>r</sup>	This study
$\Delta$ PXO_02374 mutant	PXO_02374 gene deletion mutant derived from PXO99 <sup>A</sup> , Gm <sup>r</sup>	This study
$\Delta$ PXO_02715 mutant	PXO_02715 gene deletion mutant derived from PXO99 <sup>A</sup> , Gm <sup>r</sup>	11
<b>Plasmids</b>		
pColdSUMO	Expression vector to generate an N-terminal SUMO-His <sub>6</sub> tag, Ap <sup>r</sup>	Haigene
pK18mobsacB	Suicidal vector carrying <i>sacB</i> gene for mutagenesis, Km <sup>r</sup>	29
pBBR1MCS-4	Broad-host-range expression vector, Ap <sup>r</sup>	30
pMD18-T	Cloning vector, Ap <sup>r</sup>	TaKaRa
pBlueGFP1	Plasmid containing the GFP coding sequence, Ap <sup>r</sup>	55
pMDGFP	pMD18-T containing the GFP coding sequence, Ap <sup>r</sup>	This study
pBBG	pBBR1MCS-4 containing the GFP coding sequence, Ap <sup>r</sup>	This study
pC49	pColdSUMO containing the PXO_00049 coding sequence, Ap <sup>r</sup>	This study
pC2374	pColdSUMO containing the PXO_02374 coding sequence, Ap <sup>r</sup>	This study
pC49R <sup>141</sup> D	pColdSUMO containing the point mutant of R <sup>141</sup> D in PXO_00049 sequence, Ap <sup>r</sup>	This study
pC2374R <sup>10</sup> D	pColdSUMO containing the point mutant of R <sup>10</sup> D in PXO_02374 sequence, Ap <sup>r</sup>	This study
pK49G	pK18mobSacB with PXO_00049 inserted by Gm resistance gene, Km <sup>r</sup> Gm <sup>r</sup>	This study
pK2374G	pK18mobSacB with PXO_02374 inserted by Gm resistance gene, Km <sup>r</sup> Gm <sup>r</sup>	This study
pB49	pBBR1MCS-4 carrying the PXO_00049 coding sequence, Ap <sup>r</sup>	This study
pB2374	pBBR1MCS-4 carrying the PXO_02374 coding sequence, Ap <sup>r</sup>	This study
pB49 R <sup>141</sup> D	pBBR1MCS-4 carrying the PXO_00049 coding sequence, Ap <sup>r</sup>	This study
pB2374 R <sup>10</sup> D	pBBR1MCS-4 carrying the PXO_02374 coding sequence, Ap <sup>r</sup>	This study
pBBG49	pBBG carrying the PXO_00049 coding sequence, Ap <sup>r</sup>	This study
pBBG2374	pBBG carrying the PXO_02374 coding sequence, Ap <sup>r</sup>	This study
pBBG2715	pBBG carrying the PXO_02715 coding sequence, Ap <sup>r</sup>	This study

cells were collected by centrifugation and resuspended in sterile distilled water at a concentration with an optical density at 600 nm (OD<sub>600</sub>) of 0.8. The bacterial suspensions were clip inoculated on leaves of approximately 6-week-old rice plants of the susceptible rice variety (*Oryza sativa* subsp.  *japonica*) under relevant conditions (33). For the biosafety of the genetically modified *X. oryzae* pv. *oryzae* strains, the pathogenicity tests were conducted in separate and dedicated greenhouse rooms, where the *X. oryzae* pv. *oryzae*-inoculated rice plants were strictly isolated and contained. After the completion of the experiments, all of the tested rice plants were collected and autoclaved. Pictures were taken using a digital camera (Canon EOS 550D), and disease lesion lengths were measured 14 days after inoculation.

**Motility assay.** Fresh colonies from PSA plates were stabbed into plates composed of 0.03% (wt/vol) Bacto peptone, 0.03% yeast extract, and 0.3% agar to detect swimming motility (34) and an SB medium plate containing 0.5% (wt/vol) Bacto peptone, 0.5% yeast extract, 0.5% sugar, 0.1% L-glutamic acid, and 0.6% agar to detect sliding motility (35). The inoculated cells were cultured for 4 days at 28°C and examined for bacteria swimming and sliding away from the inoculated site. The diameter of each swimming or sliding zone was measured, and its dimensions were calculated. The numbers represented the average from at least three independent repeats.

**EPS production assay.** For analyzing EPS production, bacterial strains were cultured in M210 medium for 48 h. Supernatants were collected by centrifugation at 12,000 × g for 10 min at room temperature. Two volumes of absolute ethanol was added to the supernatants. The mixtures were held at -20°C for half an hour and centrifuged to collect the precipitated EPS molecules.

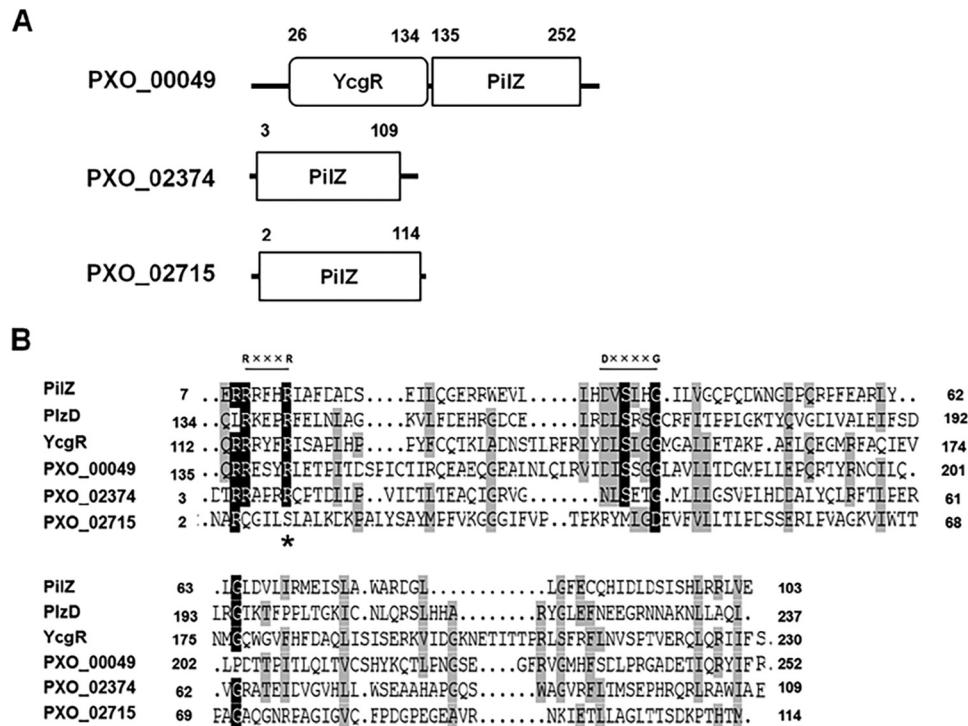
After drying at 55°C overnight, EPS molecules were weighed. The experiments were repeated three times (36).

**Biofilm formation assay.** Bacterial strains were grown to log phase in M210 medium, and then the bacteria were diluted to an OD<sub>600</sub> of 0.002 using L medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.001% glucose, and 5 ml was incubated at 28°C for 72 h in the tubes. Bacterial pellicles were stained by gently pouring off the medium, washing twice with water, and staining with 0.1% crystal violet. Tubes were washed and rinsed with water until all unbound dye was removed. The adherent dye was later redissolved with 90% ethanol and quantified by spectrophotometry at A<sub>490</sub> (37).

**RNA isolation and qRT-PCR analysis.** The expression of T3SS-related genes in *X. oryzae* pv. *oryzae* strains was induced by culture in the medium XOM2 (38). A bacterial RNAout (Tiandz) kit was used for total RNA isolation according to the manufacturer's instructions. Total RNA was treated with DNase I (Tiandz) to remove any DNA contamination. Five micrograms of RNA was used for cDNA synthesis with the random hexanucleotide primers. Quantitative reverse transcription-PCR (qRT-PCR) analysis was carried out using iQTM SYBR Green Supermix (Bio-Rad) and the gene-specific primers (see Table S1 in the supplemental material) with cDNAs as the template. The signals during amplification were detected by the iCyclerIQ real-time detection system (Bio-Rad). The relative levels of gene expression were determined by using the threshold cycle (2<sup>- $\Delta\Delta$ CT</sup>) method (39) with the DNA gyrase subunit B gene (*gyrB*) as the internal control (40). Three technical replicates were used each time.

**Localization of PilZ-GFP fusion proteins.** The green fluorescent protein (GFP) was amplified using the primers GFPF and GFPR from the





**FIG 1** Domain organization and sequence alignment of PilZ domain proteins in *Xanthomonas oryzae* pv. *oryzae* wild-type strain PXO99<sup>A</sup>. (A) Schematic representation of the domain organization of three PilZ domain proteins from *Xanthomonas oryzae* pv. *oryzae* strain PXO99<sup>A</sup>. The numbers represent amino acid residues where the predicted domains start and end, based on NCBI's conserved domain database and the SMART database. The PilZ domain and YcgR domain are shown. (B) Sequence alignment of the PilZ domain of PXO\_00049, PXO\_02374, and PXO\_02715 with PilZ (PA4608) from *P. aeruginosa*, PilzD (VCA0042) from *V. cholerae*, and YcgR from *E. coli*, which represent the conserved PilZ domain. Black and gray highlighting show amino acid residues with  $\geq 75\%$  and  $\geq 50\%$  homology, respectively. An asterisk highlights the residue confirmed by isothermal titration calorimetry assays to be involved in c-di-GMP binding.

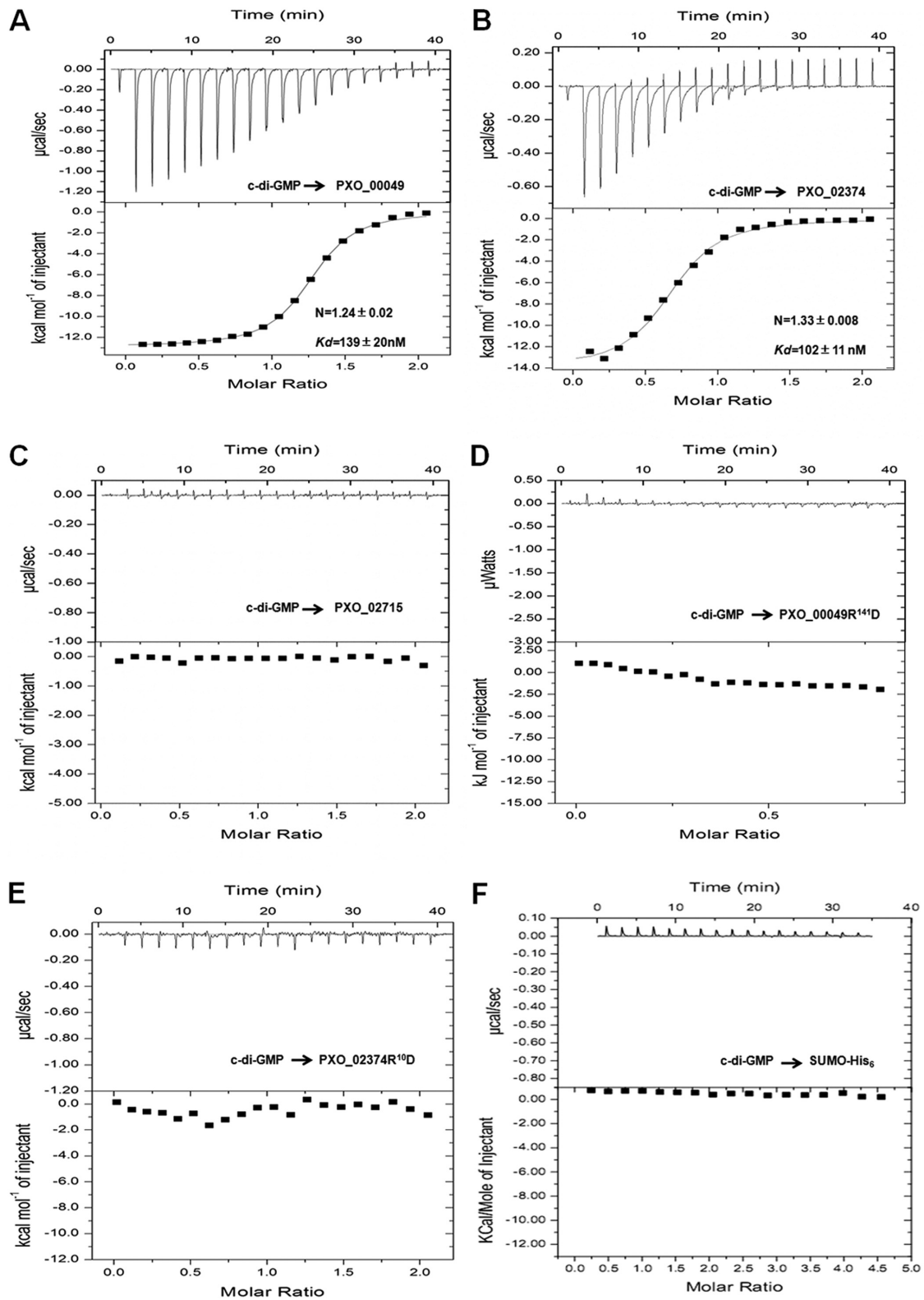
vector pBlueGFP1, and the PCR fragments were cloned into the vector pMD18-T for sequencing. We constructed pBBG by digesting an XbaI/SacI GFP fragment from pMDGFP into the vector pBBR1MCS-4. This allows the GFP fusions to be constitutively expressed by the *lac* promoter from pBBR1MCS-4. PCR primers 49CF/49LR, 2374CF/2374LR, and 2715LF/2715LR were used to amplify the fragments *PXO\_00049*, *PXO\_02374*, and *PXO\_02715*, respectively. All the coding sequences were without the termination codon. The PCR products were cloned into pMD18-T, and the fragments digested with KpnI/HindIII were subcloned into pBBG, resulting in pBBG49, pBBG2374, and pBBG2715. The plasmids were electroporated into PXO99<sup>A</sup> or mutant strains. The locations of different proteins in *X. oryzae* pv. *oryzae* cells were detected by fluorescence microscopy (Olympus BX61), and the CellSens Dimension software was used for analysis of the average brightness value of the selected area in bacterial cells.

## RESULTS

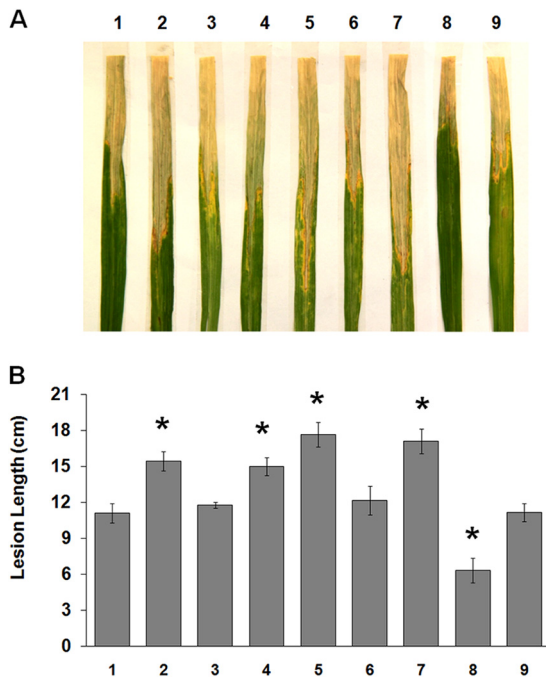
**Analysis of PilZ domain proteins in the genome of *X. oryzae* pv. *oryzae*.** Bioinformatics analysis using BLAST indicates that three genes, *PXO\_00049*, *PXO\_02374*, and *PXO\_02715*, encoding typical PilZ domains, are found in the genome of *X. oryzae* pv. *oryzae* strain PXO99<sup>A</sup>. The domain architectures of the proteins were analyzed by the simplified modular architecture research tool (SMART) online service. *PXO\_00049* is a YcgR-like protein containing an N-terminal YcgR domain, which is a predicted domain involved in regulating flagellar rotation (15, 18), and a C-terminal PilZ domain, which is a c-di-GMP-binding domain. *PXO\_02374* and *PXO\_02715* contain a single PilZ domain (Fig. 1A). Alignment of the PilZ domain protein sequences re-

vealed that the RxxxR and N/DxxxxG motifs were highly conserved in *PXO\_00049* and *PXO\_02374*, while those in *PXO\_02715* were less well conserved due to the changes to QxxxS and RxxxxD (Fig. 1B).

**c-di-GMP binding efficiency of PilZ domain proteins.** To determine whether three *X. oryzae* pv. *oryzae* proteins with PilZ domains function as receptors for c-di-GMP, we performed isothermal titration calorimetry (ITC) to detect the c-di-GMP binding affinity of these proteins. SUMOHis<sub>6</sub>-*PXO\_00049*, SUMOHis<sub>6</sub>-*PXO\_02374*, and SUMOHis<sub>6</sub>-*PXO\_02715* were titrated with an injected solution of c-di-GMP at 20°C, and the heat released upon binding was measured by the integration of power values over a range of ligand concentrations. The results showed that *PXO\_00049* and *PXO\_02374* bound to c-di-GMP in a 1:1 stoichiometry, with estimated dissociation constants ( $K_d$ ) of  $139 \pm 20$  nM and  $102 \pm 11$  nM, respectively (Fig. 2A and B). In contrast, *PXO\_02715* did not bind to c-di-GMP (Fig. 2C), and no physical interaction was observed between the control protein SUMOHis<sub>6</sub> and c-di-GMP molecule (Fig. 2F). The R residue in the RxxxR motif has been identified as the crucial site for the PilZ domain protein to bind c-di-GMP (15). To further characterize the importance of the PilZ domain for *PXO\_00049* and *PXO\_02374*, we chose R<sup>141</sup> of *PXO\_00049* and R<sup>10</sup> of *PXO\_02374* to introduce point mutations within the proteins and test their ability to bind c-di-GMP. Results from ITC assays showed that *PXO\_00049*R<sup>141</sup>D and *PXO\_02374*R<sup>10</sup>D lost their ability to bind c-di-GMP (Fig. 2D and E). These observations indicate that both



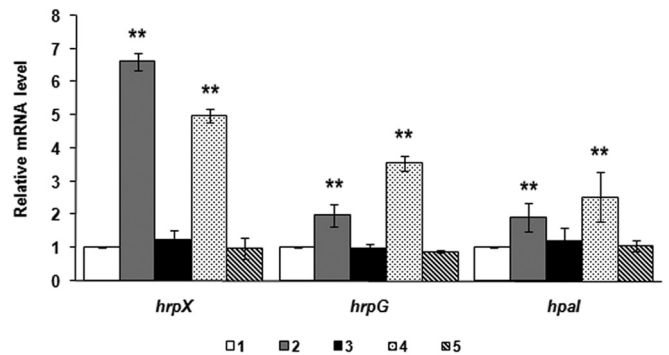
**FIG 2** Analysis of c-di-GMP binding of PilZ domain proteins using isothermal titration calorimetry assays. The top panel in each panel shows the titration calorimetry of 20  $\mu\text{M}$  SUMO-His<sub>6</sub>, 30  $\mu\text{M}$  SUMO-His<sub>6</sub>-PXO\_00049, and 50  $\mu\text{M}$  SUMO-His<sub>6</sub>-PXO\_02374 or SUMO-His<sub>6</sub>-PXO\_02715 protein with 2- $\mu\text{l}$  aliquots of 200  $\mu\text{M}$ , 30  $\mu\text{M}$ , or 500  $\mu\text{M}$  c-di-GMP at 20°C. The derived values for  $K_d$  and the N value for the binding stoichiometry of c-di-GMP to PilZ domain proteins are shown in the bottom panel. c-di-GMP binding to the recombinant proteins SUMO-His<sub>6</sub>-PXO\_00049 (A), SUMO-His<sub>6</sub>-PXO\_02374 (B), and SUMO-His<sub>6</sub>-PXO\_02715 (C); to the point mutant for R<sup>141</sup> of recombinant protein SUMO-His<sub>6</sub>-PXO\_00049 (D) and the point mutant for R<sup>10</sup> of recombinant protein SUMO-His<sub>6</sub>-PXO\_02374 (E); and to the control protein SUMO-His<sub>6</sub> (F).



**FIG 3** The PilZ domain proteins affected the virulence of *X. oryzae* pv. *oryzae* in rice. (A) *X. oryzae* pv. *oryzae* strains were inoculated by using the rice leaf clipping method. The disease symptoms were observed at 14 days after inoculation. (B) The lesion lengths were recorded. The error bars represent standard deviations of the lesion lengths from 10 leaves. Three independent experiments were performed with similar results. Lanes for both panels: 1, wild-type PXO99<sup>A</sup>; 2,  $\Delta$ PXO\_00049 mutant; 3,  $\Delta$ PXO\_00049 (pB49) mutant; 4,  $\Delta$ PXO\_00049 (pB49R<sup>141D</sup>) mutant; 5,  $\Delta$ PXO\_02374 mutant; 6,  $\Delta$ PXO\_02374 (pB2374) mutant; 7,  $\Delta$ PXO\_02374 (pB2374R<sup>10D</sup>) mutant; 8,  $\Delta$ PXO\_02715 mutant; 9,  $\Delta$ PXO\_02715 (pB2715) mutant. Asterisks indicate *P* values of <0.05 by *t* test.

PXO\_00049 and PXO\_02374 bind c-di-GMP with high affinity through the PilZ domain.

**PilZ domain proteins regulate *X. oryzae* pv. *oryzae* virulence in rice.** PilZ domain proteins have been identified as c-di-GMP receptors to perform biological functions, including virulence in diverse bacteria (4, 41). In *X. oryzae* pv. *oryzae*, deletion of the PXO\_02715 gene resulted in attenuated virulence on rice, implying that PXO\_02715 is required for the full virulence of *X. oryzae* pv. *oryzae* (11). To further characterize whether PXO\_00049 and PXO\_02374 affect the virulence, we constructed deletion mutants of the *X. oryzae* pv. *oryzae* wild-type strain PXO99<sup>A</sup>. The bacterial cells of PXO99<sup>A</sup> and derived mutants were inoculated onto the tips of the leaves as described in Materials and Methods. The disease symptoms were recorded by photography, and the lesion lengths were measured 14 days postinoculation. As shown in Fig. 3,  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants caused more severe disease symptoms and longer lesions than did the wild-type strain, suggesting that their ability to infect the host rice plant was significantly increased. When a plasmid to express PXO\_00049 and PXO\_02374 in *trans* was transformed into the relevant mutants, the disease phenotypes were restored to near-wild-type levels. In contrast, expression of PXO\_00049R<sup>141D</sup> in the  $\Delta$ PXO\_00049 mutant and PXO\_02374R<sup>10D</sup> in the  $\Delta$ PXO\_02374 mutant failed to complement its virulence, indicating that the PilZ domains of PXO\_00049 and PXO\_02374 were necessary for bacterial viru-

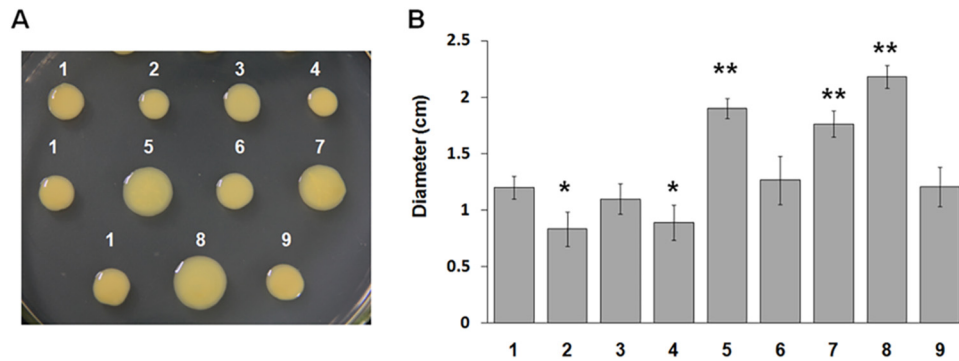


**FIG 4** Expression of *hrp* genes was upregulated in  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants as revealed by using qRT-PCR analysis. The expression level of each gene (*hrpG*, *hrpX*, and *hpa1*) in the wild-type strain PXO99<sup>A</sup> was mathematically designated 1 for each reaction. The housekeeping gene *gyrB* was used as a reference gene. Error bars represent standard deviations from three biological repeats. Bars: 1, wild-type PXO99<sup>A</sup>; 2,  $\Delta$ PXO\_00049 mutant; 3,  $\Delta$ PXO\_00049 (pB49) mutant; 4,  $\Delta$ PXO\_02374 mutant; 5,  $\Delta$ PXO\_02374 (pB2374) mutant. \*\*, *P* values of <0.01 by *t* test.

lence. These findings demonstrate that PilZ domain proteins PXO\_00049 and PXO\_02374 regulate virulence of *X. oryzae* pv. *oryzae*.

**PilZ domain proteins affect T3SS-related gene transcription.** Previous studies have shown that the PilZ domain protein PXO\_02715 is required for the full virulence of *X. oryzae* pv. *oryzae* and that the expression of T3SS-related genes is regulated by PXO\_02715 (11). Since the PXO\_00049 and PXO\_02374 mutants displayed increased virulence on rice, the T3SS expression might be speculated to be affected as well. Therefore, we examined the transcriptional levels of T3SS regulator genes *hrpG* and *hrpX* and harpin gene *hpa1* in the  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants by qRT-PCR experiments. The total RNA was extracted from the wild-type PXO99<sup>A</sup> and relevant mutants after the expression of the *hrp* genes was induced in the minimal medium XOM2 (38). As shown in Fig. 4, the mRNA levels of *hrpX*, *hrpG*, and *hpa1* in  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants increased about 1- to 6-fold compared with those in wild-type PXO99<sup>A</sup>, respectively. Complementation of  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants by expression in *trans* of PXO\_02374 or PXO\_00049 restored the gene expression to near-wild-type levels. This is consistent with the virulence phenotypes on rice of the  $\Delta$ PXO\_00049 and  $\Delta$ PXO\_02374 mutants described above. These observations suggest that both PXO\_00049 and PXO\_02374 have a negative effect on the T3SS gene expression of *X. oryzae* pv. *oryzae*.

**PilZ domain proteins modulate *X. oryzae* pv. *oryzae* sliding motility.** Recent studies have uncovered that c-di-GMP receptors regulate the twitching motility in *E. coli* and *P. aeruginosa* (15, 42, 43). To characterize the effect of three PilZ domain proteins on motility in *X. oryzae* pv. *oryzae*, the wild-type strain and relevant mutants were cultured on semisolid plates containing 0.3% agar for detection of swimming motility (34). The  $\Delta$ PXO\_00049,  $\Delta$ PXO\_02374, and  $\Delta$ PXO\_02715 mutants showed the same swimming diameter as that of the wild-type strain PXO99<sup>A</sup> (see Fig. S1 in the supplemental material). It has been reported previously that some PilZ proteins regulate the sliding or gliding motility in *X. campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *citri* (22, 35). Therefore, we tested the sliding motility of *X. oryzae* pv. *oryzae* strains on SB medium containing 0.6% agar, which has



**FIG 5** PXO\_00049, PXO\_02374, and PXO\_02715 regulate sliding motility in PXO99<sup>A</sup>. *X. oryzae* pv. *oryzae* strains were inoculated on SB plates containing 0.6% agar at 28°C for 4 days. (A) Results of a representative motility assay. (B) Bar graph represents the mean sliding diameter and standard deviation for three independent replicates. Plates (A) and bars (B): 1, PXO99<sup>A</sup>; 2, ΔPXO\_00049 mutant; 3, ΔPXO\_00049 (pB49) mutant; 4, ΔPXO\_00049 (pB49R<sup>141</sup>D) mutant; 5, ΔPXO\_02374 mutant; 6, ΔPXO\_02374 (pB2374) mutant; 7, ΔPXO\_02374 (pB2374R<sup>10</sup>D) mutant; 8, ΔPXO\_02715 mutant; 9, ΔPXO\_02715 (pB2715) mutant. \* and \*\*, *P* values of <0.05 and 0.01, respectively, by *t* test.

been previously used to analyze the sliding motility of other *Xanthomonas* strains (11, 33). As shown in Fig. 5, the ΔPXO\_00049 mutant showed a smaller sliding zone than that of the wild-type strain PXO99<sup>A</sup>, and the diameter decreased to approximately 2 mm, whereas the sliding zones of the ΔPXO\_02374 and ΔPXO\_02715 mutants were bigger than that of PXO99<sup>A</sup>. Expression in *trans* of the full-length open reading frames (ORFs) PXO\_00049, PXO\_02374, and PXO\_02715 in the mutants restored sliding motility to near-wild-type levels. Moreover, expression in *trans* of PXO\_00049R<sup>141</sup>D in the ΔPXO\_00049 mutant and of PXO\_02374R<sup>10</sup>D in the ΔPXO\_02374 mutant failed to complement its sliding motility. These results indicate that three PilZ domain proteins differentially regulate sliding motility rather than swimming motility in *X. oryzae* pv. *oryzae*.

**PilZ domain proteins do not control EPS production and biofilm formation.** Based on the observation that PilZ domain proteins are required for bacterial virulence of *X. oryzae* pv. *oryzae*, we further investigated whether PXO\_00049, PXO\_02374, and PXO\_02715 affect the production of virulence factors of *X. oryzae* pv. *oryzae*. EPS production and biofilm formation in the wild-type strain PXO99<sup>A</sup> and derived mutants were examined. No difference was detected between PXO99<sup>A</sup> and the three mutants in terms of EPS production by quantification (see Fig. S1A in the supplemental material) and colony surface checking (see Fig. S1B), indicating that PXO\_00049, PXO\_02374, and PXO\_02715 did not regulate the EPS production of *X. oryzae* pv. *oryzae*. Similarly, results from the biofilm formation assays showed that there are no changes between the wild-type and relevant mutants as well (see Fig. S1E). These observations suggest that PilZ domain proteins are not involved in the regulation of both EPS production and biofilm formation in *X. oryzae* pv. *oryzae*.

**Subcellular localizations of PilZ domain proteins in *X. oryzae* pv. *oryzae*.** Since specific subcellular localization of a given protein might be required for its proper biological function in bacteria (44–46), we analyzed the subcellular localization of the *X. oryzae* pv. *oryzae* PilZ proteins by constructing C-terminal translational fusions of GFP to PXO\_00049, PXO\_02374, and PXO\_02715. The restoration of sliding motility in the relevant mutants with transformed fusion proteins to the wild-type level was observed (data not shown), demonstrating that the fusion proteins retained their function. The wild-type strain PXO99<sup>A</sup>,

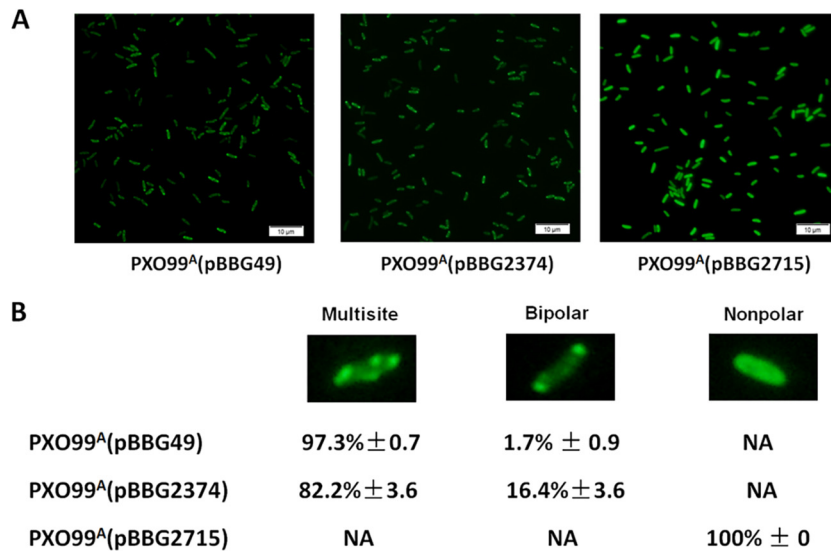
which contained the plasmid pBBG (empty vector), showed very low fluorescence, with average brightness values below 60. Subcellular localization of the PilZ domain proteins was defined based on the average brightness value in different sites within the cytoplasm of the bacterium (see Fig. S2 in the supplemental material). As shown in Fig. 6, PXO\_00049-GFP and PXO\_02374-GFP proteins displayed multisite distributions of 97.3% and 82.2%, respectively, of all bacterial cells tested, while PXO\_02715-GFP showed nonpolar localization with high-level fluorescence in 100% of cells. These results demonstrate that both PXO\_00049 and PXO\_02374 proteins mainly show similar multisite localization, whereas PXO\_02715 protein displays nonpolar distribution in the *X. oryzae* pv. *oryzae* cells.

## DISCUSSION

PilZ domain proteins are widely distributed in bacterial genomes (5). Several PilZ domain proteins have been reported to bind c-di-GMP signal molecules and function as the receptors in mediating c-di-GMP signaling and regulation of downstream phenotypes in pathogenic bacteria (20, 21, 47, 48). In this work, we comparatively characterized three PilZ domain proteins (PXO\_00049, PXO\_02374, and PXO\_02715) encoded in the genome of *X. oryzae* pv. *oryzae* wild-type strain PXO99<sup>A</sup>. The observation that c-di-GMP can be effectively bound to PXO\_00049 and PXO\_02374, but not PXO\_02715 (Table 2), might be due to the fact that c-di-GMP binding motifs in PilZ domains are conserved in PXO\_00049 and PXO\_02374 but are degenerate in PXO\_02715 (Fig. 1). For PXO\_00049 and PXO\_02374, point mutations in the R<sup>141</sup> and R<sup>10</sup> residues within the PilZ domains completely abolish the c-di-GMP binding ability, confirming the requirement of the PilZ domain for c-di-GMP binding. For PXO\_02715 showing no direct c-di-GMP binding activity, specific interaction with the EAL domain of the c-di-GMP receptor Filp was observed (unpublished data), implying that c-di-GMP sensing by Filp is required for its proper function in *X. oryzae* pv. *oryzae*.

*X. oryzae* pv. *oryzae* has been one of the most important bacterial pathogens of rice, leading to 20 to 50% yield losses, and has become an ideal model to study the molecular interaction between plant and pathogenic bacterium (23). It is of both scientific and economic significance to decipher the regulatory mechanisms in





**FIG 6** Subcellular localization of PilZ domain proteins in PXO99<sup>A</sup>. *X. oryzae* pv. *oryzae* strains were cultured in M210 at 28°C until reaching an OD<sub>600</sub> of 0.6. (A) Photograph of the GFP fusion protein in *X. oryzae* pv. *oryzae* strains detected using a fluorescence microscope (Olympus BX61). (B) Multisite, bipolar, and nonpolar locations were defined as three types for subcellular localization of the proteins. The different subcellular locations of PilZ domain proteins in PXO99<sup>A</sup>(pBBG49), PXO99<sup>A</sup>(pBBG2374), and PXO99<sup>A</sup>(pBBG2715) strains were counted. ± represents standard deviations from three biological repeats. NA, not available.

virulence of *X. oryzae* pv. *oryzae*. c-di-GMP receptors have been identified to play important roles in the regulation of virulence (49), while the regulatory functions of PilZ domain proteins in virulence expression were examined in a few pathogenic bacteria. For example, gene deletion of the PilZ domain protein from *V. cholerae* resulted in reduced colonization of the infant mouse small intestine (21). Mutation of *X. campestris* pv. *campestris* PilZ domain genes *XC\_0965* and *XC\_3221*, but not *XC\_2317*, led to significantly reduced virulence in Chinese radish, suggesting their role in virulence (22). The *X. oryzae* pv. *oryzae* PilZ protein PXO\_02715, which is a homologue of XC3221, was previously shown to be an interactor with the c-di-GMP receptor FilP and to be required for the full virulence of *X. oryzae* pv. *oryzae* and regulated the expression of T3SS-related genes (11). These results mentioned above demonstrated that mutations of the PilZ domain proteins led generally to bacterial virulence reduction. However, the PilZ domain proteins PXO\_00049 and PXO\_02374 were identified in this study as the novel negative regulators of virulence and T3SS-related gene expression in *X. oryzae* pv. *oryzae* (Table 2). A few negative bacterial regulators in the c-di-GMP regulation of virulence expression have been reported, such as CgsB (a c-di-GMP synthase) in a mouse model of *Brucella melitensis* infection (50). To our knowledge, this is the first report that deletion of the PilZ-type protein gene produces a hypervirulent strain of pathogenic bacteria. Nevertheless, it remains to be determined what is the exact molecular mechanism by which the PilZ-type receptors affect bacterial virulence gene expression. In addition, based on the observation that the PilZ domain proteins with high similarities to *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* demonstrated either common or distinctive regulatory roles in virulence, we assume that their mechanistic role in virulence varies in the two species. Moreover, changes in the R<sup>141</sup> and R<sup>10</sup> residues within the PilZ domain of PXO\_00049 and PXO\_02374 proteins, respectively, resulted not only in a loss of c-di-GMP binding abil-

ity but also in complementation of the relevant phenotypes for virulence restoration in the mutant strains. This indicates that c-di-GMP binding via the PilZ domain is necessary for PXO\_00049 and PXO\_02374 to function properly *in vivo*. Moreover, expression of T3SS-related *hrp* genes was regulated by all PilZ domain proteins in the same ways as they exerted a virulence phenotype in rice (11) (Fig. 3). However, three PilZ proteins were all not involved in both EPS production and biofilm formation in *X. oryzae* pv. *oryzae*, which was not the case reported for *X. campestris* pv. *campestris* (22). Taken together, our data provide novel insights into the mechanisms underlying the PilZ protein-mediated c-di-GMP regulation of virulence via T3SS expression in *X. oryzae* pv. *oryzae*.

It has been reported elsewhere that PilZ domain proteins are involved in the regulation of swimming motility in *E. coli*, *Salmonella*, and *Pseudomonas fluorescens* (15–18, 20) and sliding or gliding but not swimming motility in *Xanthomonas* spp. (11, 22, 35). These observations implicate differential regulation by PilZ proteins of bacterial motility in different species. Sliding motility is the action of sliding or gliding on a semisolid surface and, in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* strains, is

**TABLE 2** Functional characterization of three PilZ domain proteins in *Xanthomonas oryzae* pv. *oryzae*<sup>a</sup>

Strain	c-di-GMP binding/ dissociation constant ( $K_d$ )	Virulence	Sliding motility	Subcellular location
PXO_00049	Yes/139 ± 20 nM	↑	↓	Multisite
PXO_02374	Yes/102 ± 11 nM	↑	↑	Multisite/ bipolar
PXO_02715	No/NA	↓	↑	Nonpolar

<sup>a</sup> ↑ and ↓, increased and decreased virulence or sliding motility of the relevant mutant compared with the wild type, respectively. NA, not available.



recognized to be dependent on the bacterial pilus (22, 35). In addition, type IV pili (T4P) have also been shown to be involved in virulence of pathogenic bacteria, including *P. aeruginosa*, *Neisseria meningitidis*, and *Ralstonia solanacearum* (51–53). In this study, the PilZ proteins were found to regulate sliding motility, among which PXO\_00049 acts as a positive regulator, whereas both PXO\_02374 and PXO\_02715 function as the negative ones. However, where and how PilZ domain proteins affect pilus biogenesis, and what the relationship is between the T4P and virulence in *X. oryzae* pv. *oryzae*, remain to be further elucidated.

Protein subcellular localization is generally thought to be related to specific spatial functions in bacteria (44–46). In this study, the characteristics of PilZ domain protein localizations showed multisite localization of both PXO\_00049 and PXO\_02374 and nonpolar distribution of PXO\_02715 in the *X. oryzae* pv. *oryzae* cells (Fig. 6). These results suggest that there might be a possible link among c-di-GMP binding, virulence, gene expression, and subcellular localization for the *X. oryzae* pv. *oryzae* PilZ domain proteins. Therefore, this provides informative clues for further exploration of the mechanistic regulation by the PilZ domain proteins of bacterial virulence and pathogenesis in rice.

For PilZ domain proteins, the interaction with downstream proteins has been identified as a major way to perform biological functions, including bacterial virulence and motility (17, 18, 54). For example, YcgR decreases bacterial swimming speed by directly binding to the flagellar motor component at high c-di-GMP levels, which is a signal for switching into a biofilm-associated lifestyle (15–18). The *X. axonopodis* pv. *citri* PilZ domain protein PilZ<sub>XAC1133</sub> and a PilB ATPase formed a complex to promote T4P formation and inhibit sliding motility (35, 53). The *X. oryzae* pv. *oryzae* PilZ domain protein PXO\_02715 showed protein sequence similarity of 94.4% to PilZ<sub>XAC1133</sub>, suggesting that its regulation of virulence and motility might be occurring by interacting with proteins involved with T4P formation. In addition, two novel PXO\_02374-interacting proteins have been identified through yeast two-hybrid and glutathione S-transferase (GST) pulldown assays (unpublished data). We speculate that the interactions between the PilZ domain proteins and downstream proteins might occur and that the intracellular concentrations of c-di-GMP affect such protein-protein interactions and their mediation of downstream signaling mechanism, thereby regulating T3SS and virulence expression of *X. oryzae* pv. *oryzae*. Further identification and characterization of PilZ domain protein interaction, and their regulation of virulence or motility, are required to elucidate mechanisms by which PilZ domain proteins exert their biological functions in *X. oryzae* pv. *oryzae*.

## ACKNOWLEDGMENTS

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