Phosphodiesterase EdpX1 Promotes *Xanthomonas oryzae* pv. *oryzae* Virulence, Exopolysaccharide Production, and Biofilm Formation

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ABSTRACT In *Xanthomonas oryzae* pv. *oryzae*, the bacterial blight pathogen of rice, there are over 20 genes encoding GGDEF, EAL, and HD-GYP domains, which are potentially involved in the metabolism of second messenger c-di-GMP. In this study, we focused on the characterization of an EAL domain protein, EdpX1. Deletion of the *edpX1* gene resulted in a 2-fold increase in the intracellular c-di-GMP levels, which were restored to the wild-type levels in the complemented $\Delta edpX1$ (pB-*edpX1*) strain, demonstrating that EdpX1 is an active phosphodiesterase (PDE) in *X. oryzae* pv. *oryzae*. In addition, colorimetric assays further confirmed the PDE activity of EdpX1 by showing that the E153A mutation at the EAL motif strongly reduced its activity. Virulence assays on the leaves of susceptible rice showed that the $\Delta edpX1$ mutant was severely impaired in causing disease symptoms. In *trans* expression of wild-type *edpX1*, but not *edpX1*^{E153A}, was able to complement the weakened virulence phenotype. These results indicated that an active EAL domain is required for EdpX1 to regulate the virulence of *X. oryzae* pv. *oryzae*. We then demonstrated that the $\Delta edpX1$ mutant was defective in secreting exopolysaccharide (EPS) and forming biofilms. The expression of *edpX1* in the $\Delta edpX1$ mutant, but not *edpX1*^{E153A}, restored the defective phenotypes to near-wild-type levels. In addition, we observed that EdpX1-green fluorescent protein (EdpX1-GFP) exhibited multiple subcellular localization foci, and this pattern was dependent on its transmembrane (TM) region, which did not seem to directly contribute to the regulatory function of EdpX1. Thus, we concluded that EdpX1 exhibits PDE activity to control c-di-GMP levels, and its EAL domain is necessary and sufficient for its regulation of virulence in *X. oryzae* pv. *oryzae*.

IMPORTANCE Bacteria utilize c-di-GMP as a second messenger to regulate various biological functions. The synthesis and degradation of c-di-GMP are catalyzed by GGDEF domains and an EAL or HD-GYP domain, respectively. Multiple genes encoding these domains are often found in one bacterial strain. For example, in the genome of *X. oryzae* pv. *oryzae* PXO99A, 26 genes encoding proteins containing these domains were identified. Therefore, to fully appreciate the complexity and specificity of c-di-GMP signaling in *X. oryzae* pv. *oryzae*, the enzymatic activities and regulatory functions of each GGDEF, EAL, and HD-GYP domain protein need to be elucidated. In this study, we showed that the EAL domain protein EdpX1 is a major PDE to regulate diverse virulence phenotypes through the c-di-GMP signaling pathway.

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Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight disease on rice, one of the most devastating diseases of this staple crop (1). Under disease-favorable conditions, such as high humidity, this pathogen can cause yield losses of up to 70% in susceptible rice cultivars (2). Typically, *X. oryzae* pv. *oryzae* cells enter rice leaves through wounds or hydathodes and then invade the xylem vessels, leading to systemic infection (1). The entire plant could be killed if the infection occurs at the seedling stage, while the whole leaf becomes dry and eventually dies if an older plant is infected.

Many virulence factors have been revealed to contribute to the pathogenesis of *X. oryzae* pv. *oryzae* at different stages. Adhesion-like proteins facilitate bacterial attachment and entry into the leaves (3). Exopolysaccharide (EPS) production and biofilm formation are important for bacterial colonization in the xylem vessels and for symptom development (4, 5). CatB catalase is critical for *X. oryzae* pv. *oryzae* to remove reactive oxygen species and maintain redox balance (6, 7). The type II secretion system (T2SS) and the secreted enzymes help bacterial cells degrade plant cell walls (8, 9). At last, the type III secretion system (T3SS) and its effectors play a key role in determining the consequences of the interactions between *X. oryzae* pv. *oryzae* and the rice host (9–11). Diverse regulators, including transcriptional factors, two-component systems, and diffusible signal factor (DSF) signaling pathways control the expression of the virulence factors in a cooperative manner and contribute significantly to the virulence of *X. oryzae* pv. *oryzae* *in planta* (12–16).

c-di-GMP, a ubiquitous second messenger in bacteria, controls a series of biological functions, including cell cycles, motility, biofilm formation, and the expression of virulence factors in pathogenic bacteria (17–19). Overall, a high c-di-GMP level is associated with a sessile lifestyle and biofilm formation, while a low c-di-GMP level promotes motility and virulence gene expression (17). Biochemistry and structural studies have illustrated that the metabolism of c-di-GMP is catalyzed by GGDEF domain proteins and EAL or HD-GYP domain proteins, which exhibit diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities, respectively. The changes in c-di-GMP levels are sensed by diverse groups of effectors, including PilZ domain proteins (20–23), enzymatically inactive GGDEF or EAL domains (24–27), riboswitches (28), and transcription factors (29, 30), leading to different cellular activities.

EAL domains can specifically and rapidly hydrolyze c-di-GMP into linear dimeric GMP (pGpG) (31–34). This PDE activity is dependent on the presence of the divalent cations Mn^{2+} and Mg^{2+} but is strongly inhibited by Ca^{2+} or Zn^{2+} (33, 35, 36). Crystal structures of EAL domains have revealed the presence of a triose-phosphate isomerase (TIM) barrel-like fold with one antiparallel β -strand, with the active site located at the C terminus of the barrel (24). Conserved residues involved in binding with metal ions and interacting with the substrate are critical for the catalytic activity (37).

Our lab has previously characterized several c-di-GMP-metabolic enzymes and effector proteins in *X. oryzae* pv. *oryzae*, revealing the importance and complexity of c-di-GMP signaling pathways in regulating the virulence of *X. oryzae* pv. *oryzae* (15, 38–40). There are two genes encoding single EAL domain proteins, and their enzymatic activities have not yet been confirmed. Here, we focused on PXO_03877 (here EdpX1 [EAL-domain protein of *Xoo* 1]), which contains a TM and an EAL domain. We showed that EdpX1 was an active PDE to control c-di-GMP concentrations in *X. oryzae* pv. *oryzae*, and EdpX1 regulated various virulence traits through the c-di-GMP signaling pathway.

RESULTS

EAL domain of EdpX1 contains essential residues for PDE activity. Two genes encoding single EAL domain proteins are found in the genome of *X. oryzae* pv. *oryzae* PXO99^A (15); one of them was named *edpX1*. The EdpX1 protein contains an N-terminal

FIG 1 Amino acid residues essential for PDE activity are conserved in EdpX1. Sequence alignment of the EAL domain of EdpX1 with other EAL domains, which have been confirmed to be enzymatically active, including RocR (GenBank accession no. [ONN13160.1](#)) from *Pseudomonas aeruginosa*, TBD1265 (GenBank accession no. [AAZ97218.1](#)) from *Thiobacillus denitrificans*, YahA (GenBank accession no. [AHY69061.1](#)) from *Escherichia coli*, DosP (GenBank accession no. [KYO66268.1](#)) from *Escherichia coli*, BlrP1 (GenBank accession no. [ABR77029.1](#)) from *Klebsiella pneumoniae*, VieA (GenBank accession no. [SPM21115.1](#)) from *Vibrio cholerae*, CC3396 (GenBank accession no. [AAK25358.1](#)) from *Caulobacter crescentus*, EcpC (GenBank accession no. [OOC15055.1](#)) from *Dickeya dadantii*, and PdeR (GenBank accession no. [ACD59144.1](#)) and EdpX1 (GenBank accession no. [ACD56999.1](#)) from *X. oryzae* pv. *oryzae*. The amino acids highlighted in black stand for 100% homology, and gray indicates a homology level of $\geq 75\%$. E¹⁵³, N²¹¹, E²⁴³, and D²⁷² (asterisks) are conserved residues which may bind to metal iron. The conserved loop (D²⁷³FGTGYSS²⁸¹) structure is reported to be essential for activity and for binding Mg²⁺ and c-di-GMP. E³²⁹ (filled circle) was reported to coordinate with the second Mg²⁺ and E¹⁷⁶ (open triangle) to bind with the substrate.

transmembrane domain (96 to 115 amino acids [aa]) and a C-terminal EAL domain (117 to 361 aa). Basic Local Alignment Search Tool (BLAST) searches found that the homologous proteins of EdpX1 existed in most of the sequenced *Xanthomonas* species genomes (see Fig. S1 in the supplemental material). Protein sequence alignment and the resultant phylogenetic tree indicated that EdpX1 was quite conserved within the *Xanthomonas* genus (Fig. S1). Previous studies have revealed that certain residues are critical for the EAL domain to perform c-di-GMP-specific PDE activity (37). We thus asked whether these residues are conserved in the EAL domain of EdpX1. Therefore, we performed amino acid sequence alignment with previously confirmed active EAL domains from different bacterial species (Fig. 1). The results showed that the loop structure and essential residues for PDE activity are present, suggesting that EdpX1 is very likely to be enzymatically active.

PDE activity of EdpX1 was demonstrated both *in vivo* and *in vitro*. To further investigate the putative PDE activity of EdpX1, we deleted the *edpX1* gene ($\Delta edpX1$) in

FIG 2 The PDE activity of EdpX1 was demonstrated both *in vivo* and *in vitro*. (A) Intracellular c-di-GMP was extracted from PXO99^A(pBBR), the mutant Δ edpX1(pBBR) strain, and the complemented Δ edpX1(pB-edpX1) strain, as described in Materials and Methods, and measured by LC-MS-MS method. (B) PDE activity of EdpX1 was tested by *in vitro* colorimetric method. Purified recombinant proteins EdpX1-His₆, EdpX1^{E153A}-His₆, and EdpX1^{E176A}-His₆ were incubated with 5 mM bis-pNPP in a reaction system at 37°C for 2 h. Hydrolysis of colorless bis-pNPP into yellow pNP was detected at 410 nm by spectrophotometer. Different letters (a to c) over the bars indicate a significant difference ($P < 0.05$) by the LSD test.

X. oryzae pv. *oryzae* and compared its intracellular c-di-GMP levels with those of the wild-type PXO99^A using liquid chromatography-tandem mass spectrometry (LC-MS/MS). As shown in Fig. 2A, the c-di-GMP concentration was increased by approximately 2-fold in the Δ edpX1 mutant relative to that in the wild-type bacteria. In addition, the elevated c-di-GMP concentration was restored in the complemented Δ edpX1(pB-edpX1) strain, which harbors a plasmid to express *edpX1* in *trans*. These results demonstrated that EdpX1 is an active PDE to degrade c-di-GMP in PXO99^A. To further confirm the PDE activity of EdpX1, we expressed and purified wild-type protein EdpX1-His₆, and mutagenized proteins EdpX1^{E153A}-His₆ and EdpX1^{E176A}-His₆, which carried point mutations at the EAL motif binding to metal cation, and the putative site to bind one of the two guanines (G1 base) in the substrate, respectively (37). Their enzymatic activities were tested using a colorimetric assay in which the PDE activity was indicated by the hydrolysis of colorless bis(*p*-nitrophenyl) phosphate (bis-pNPP) into yellow *p*-nitrophenol (pNP). Compared with the wild-type protein EdpX1, EdpX1^{E153A} exhibited about 30% of the wild-type PDE activity, while EdpX1^{E176A} exhibited about 70% of the wild-type PDE activity (Fig. 2B). These data clearly demonstrated that EdpX1 harbors PDE activity, and E153 appears to be more essential for this activity than E176.

PDE activity of EdpX1 is required for the full virulence of *X. oryzae* pv. *oryzae* on rice. Knowing that bacteria modulate c-di-GMP levels to regulate virulence-related phenotypes (41), we thus investigated whether *edpX1* contributes to the virulence of *X. oryzae* pv. *oryzae* on rice plants. We inoculated wild-type PXO99^A(pBBR), the mutant Δ edpX1(pBBR) strain, and its complemented Δ edpX1(pB-edpX1) strain on susceptible rice plants (*Oryza sativa* L. *japonica* cv. Nipponbare) and recorded disease phenotypes on 14 days postinoculation (dpi). The mutant Δ edpX1(pBBR) strain displayed significantly reduced virulence compared with the wild type, while in *trans* expression of the full-length *edpX1* gene using the broad-host-vector pBBR1MCS-4 was able to partially restore the disease symptoms (Fig. 3A and B). In addition, the complemented strains containing the mutated *edpX1* genes Δ edpX1(pB-edpX1^{E153A}) and Δ edpX1(pB-edpX1^{E176A}) were also constructed. The expression levels of EdpX1 and its variants in these strains were confirmed by Western blotting (Fig. S2). The virulence of the strains containing the genes Δ edpX1(pB-edpX1^{E153A}) and Δ edpX1(pB-edpX1^{E176A}) was then tested on susceptible rice plants. The complemented Δ edpX1(pB-edpX1^{E153A}) strain caused disease symptoms and lesion length similar to those of the mutant Δ edpX1(pBBR) strain, indicating that EdpX1^{E153A} was not able to perform the same function as the wild-type EdpX1. Interestingly, the lesion length of the Δ edpX1(pB-

FIG 3 *edpX1* gene contributes to the virulence of *X. oryzae* pv. *oryzae* on rice. (A) The following strains were inoculated on rice by the leaf-clipping method: wild-type PXO99^Δ(pBBR), the mutant Δ edpX1(pBBR) strain, and the complemented Δ edpX1(pB-edpX1), Δ edpX1(pB-edpX1^{E153A}), and Δ edpX1(pB-edpX1^{E176A}) strains. The disease symptoms were observed on 14 dpi. (B) The lesion lengths were recorded. The error bars represent standard deviations of the lesion lengths from 10 leaves. Different letters (a to c) over the bars indicate a significant difference ($P < 0.05$) by the LSD test. (C) Growth curves were generated by starting bacterial cultures at an OD₆₀₀ of 0.1 and then measuring the OD₆₀₀ of each strain every 6 h for a period of 72 h. (D) Bacterial numbers (CFU) were calculated for each strain when grown in M210 for 24 h, 48 h, and 72 h, respectively. Three independent experiments were performed with similar results.

edpX1^{E176A}) strain resembled that of the Δ edpX1(pB-edpX1) strain (Fig. 3A and B). Collectively, these results suggested that EdpX1 is required for the full virulence of *X. oryzae* pv. *oryzae* on rice leaves, and its PDE activity is critical for this function.

To exclude the possibility that different growth rates might cause differences in virulence, we monitored the growth status for above-mentioned strains. All cultures started at the same cell density, which was an optical density at 600 nm (OD₆₀₀) of 0.1. During the period of 72 h, no significant difference was observed between the strains (Fig. 3C). In addition, bacterial cell numbers were also calculated at 24 h, 48 h, and 72 h, and no significant difference was detected between the strains (Fig. 3D). These results support the idea that the differences observed in the virulence assay were due to the modulation of intracellular c-di-GMP levels by EdpX1 but not bacterial growth.

EdpX1 affected EPS production by regulating *gum* gene expression. EPS is one of the most important virulence factors in *X. oryzae* pv. *oryzae* (4). Since the virulence of the Δ edpX1 mutant on rice leaves was significantly reduced, we were interested in examining its EPS production levels. Therefore, we examined EPS production from various strains, including the PXO99^Δ(pBBR) strain and the Δ edpX1(pBBR), Δ edpX1(pB-edpX1), Δ edpX1(pB-edpX1^{E153A}), and Δ edpX1(pB-edpX1^{E176A}) strains. The colony of wild-type PXO99^Δ showed a smooth and shiny surface due to the production of a large

FIG 4 EdpX1 regulates EPS production through modulating *gum* gene expression in *X. oryzae* pv. *oryzae*. (A) EPS production in various strains was determined. (B) Expression of representative *gum* genes was determined in various strains by qRT-PCR assays. The expression level of each *gum* gene in wild-type strain was mathematically designated 1 for each reaction. The relative expression levels of each gene in other strains were calculated using the $2^{-\Delta\Delta CT}$ method. Test strains were indicated as different numbers, as follows: 1, PXO99^A(pBBR); 2, the $\Delta edpX1$ (pBBR) strain; 3, the $\Delta edpX1$ (pB-*edpX1*) strain; 4, the $\Delta edpX1$ (pB-*edpX1*^{E153A}) strain; and 5, the $\Delta edpX1$ (pB-*edpX1*^{E176A}) strain. Three independent experiments were performed with similar results. Different letters (a to d) over the bars indicate a significant difference ($P < 0.05$) by the LSD test.

amount of EPS. Comparatively, the colony surface of the $\Delta edpX1$ mutant was dry and flat. Quantification of EPS production showed that the deletion of *edpX1* decreased the values to approximately 40% of the wild type (Fig. 4A), which was in agreement with the colony morphology. As expected, both the colony morphology and the amount of secreted EPS of the full-length complemented $\Delta edpX1$ (pB-*edpX1*) strain were partially restored to the wild-type level. In contrast, the $\Delta edpX1$ (pB-*edpX1*^{E153A}) strain displayed an even drier colony surface and produced EPS amounts similar to that of the mutant, while the $\Delta edpX1$ (pB-*edpX1*^{E176A}) strain restored the colony morphology and EPS amount similar to that of the $\Delta edpX1$ (pB-*edpX1*) strain (Fig. 4A). These results suggested that EdpX1 positively regulated EPS production in *X. oryzae* pv. *oryzae*, and its PDE activity is required for the function.

The biogenesis of EPS is encoded by a cluster of 12 genes, *gumB* to *gumM* (42). It has been shown that not each *gum* gene contributes equally to EPS production and virulence in *X. oryzae* pv. *oryzae* (4). Although the 12 genes are transcribed in a polycistronic mRNA, internal promoters have also been located which drive the formation of other overlapping transcripts (42, 43). To further determine at which level the EPS production is affected, we performed quantitative real-time PCR (qRT-PCR) to examine the expression of *gum* genes. *gumB*, *gumD*, and *gumK* were selected, because mutations of them severely affected the production of EPS and the virulence in *X. oryzae* pv. *oryzae* (4). The transcription of *gumB* and *gumD* might be driven from the promoter upstream of *gumB*, while *gumK* might be transcribed from the internal

FIG 5 EdpX1 modulates biofilm formation in *X. oryzae* pv. *oryzae*. Biofilm formation of various strains, including PXO99A(pBBR) and the Δ edpX1(pBBR), Δ edpX1(pB-edpX1), Δ edpX1(pB-edpX1^{E153A}), and Δ edpX1(pB-edpX1^{E176A}) strains was visualized by crystal violet staining in polystyrene 96-well microplates and quantified by measuring the optical density at 490 nm after suspension in ethanol. Three independent experiments were performed with similar results. Different letters (a to c) over the bars indicate a significant difference ($P < 0.05$) by the LSD test.

promoter upstream of *gumG* (43). The expression levels of representative *gum* genes were determined in PXO99A(pBBR) and the Δ edpX1(pBBR), Δ edpX1(pB-edpX1), Δ edpX1(pB-edpX1^{E153A}), and Δ edpX1(pB-edpX1^{E176A}) strains (Fig. 4B). Compared with the wild type, the expression of *gumB*, *gumD*, and *gumK* in the Δ edpX1 mutant dropped about 90%, 40%, and 10%, respectively. In the Δ edpX1(pB-edpX1) strain, the expression level of each *gum* gene was recovered to a certain extent, while in the Δ edpX1(pB-edpX1^{E153A}) strain, the expression patterns of these genes were similar to those in the Δ edpX1 mutant but with an even lower value. The gene expression levels recovered in the Δ edpX1(pB-edpX1^{E176A}) strain were similar to those in the Δ edpX1(pB-edpX1) strain. Thus, we concluded that EdpX1 positively controls EPS production, which is partially through regulating *gum* gene expression.

EdpX1 contributes to biofilm formation of *X. oryzae* pv. *oryzae*. Due to EPS being part of biofilms, EPS usually influence the biofilm formation which helps bacterial adhere to the host cell (44). Therefore, we wanted to know whether *edpX1* affected the biofilm formation of *X. oryzae* pv. *oryzae*. Thus, we examined the biofilm formation abilities in different strains, including PXO99A(pBBR), the Δ edpX1(pBBR) strain, the Δ edpX1(pB-edpX1) strain, the Δ edpX1(pB-edpX1^{E153A}) strain, and the Δ edpX1(pB-edpX1^{E176A}) strain. The results showed that the Δ edpX1 mutant was strongly impaired in forming wild-type level biofilm, while the complemented Δ edpX1(pB-edpX1) strain restored the phenotype to near-wild-type level. The Δ edpX1(pB-edpX1^{E153A}) strain displayed a biofilm formation level similar to that of the Δ edpX1 mutant, while the Δ edpX1(pB-edpX1^{E176A}) strain displayed a phenotype similar to that of the Δ edpX1(pB-edpX1) strain (Fig. 5). Overall, the biofilm formation abilities were consistent with the EPS production levels in an EdpX1-dependent manner. In many cases, biofilm formation is also affected by bacterial motility (45). We then detected swimming motilities in the above-mentioned strains. The results showed that there were no differences between these strains (Fig. S3), suggesting that swimming motility might not contribute to the EdpX1-mediated biofilm formation abilities.

Transmembrane domain of EdpX1 determines its subcellular multisite localization but does not contribute to its regulatory function. Besides the EAL domain, EdpX1 also harbors a TM domain on its N terminus. It has been shown that the TM domains of STM2215, an EAL domain containing protein from *Salmonella enterica*

FIG 6 Subcellular localization of EdpX1 and its derivatives in *X. oryzae* pv. *oryzae*. (A) Photograph of the GFP fusion proteins EdpX1-GFP, EdpX1^{E153A}-GFP, EdpX1^{E176A}-GFP and EdpX1^{ΔTM}-GFP in *X. oryzae* pv. *oryzae* cells detected using a fluorescence microscope (Olympus BX61). (B) Representative localization patterns for each fusion protein was determined. Multisite, bipolar, and nonpolar localization patterns were observed. NA, not available.

serovar Typhimurium, facilitated the protein localization to bacterial inner membranes (34). They also showed that the localization was not related to the regulatory function of STM2215 (34). We wanted to investigate the role of the TM domain in EdpX1. Thus, we constructed plasmids containing *edpX1* or its derivatives fused to the *gfp* gene. The expression levels of EdpX1-GFP, EdpX1^{E153A}-GFP, EdpX1^{E176A}-GFP, and EdpX1^{ΔTM}-GFP in wild-type PXO99^Δ were confirmed using Western blotting (Fig. S4), and their subcellular localization patterns were observed under fluorescence microscopy. As shown in Fig. 6, most EdpX1-GFP proteins showed specific foci in *X. oryzae* pv. *oryzae* cells, while fewer than 6% of the cells showed bipolar signals. The distribution patterns of EdpX1^{E153A}-GFP and EdpX1^{E176A}-GFP in *X. oryzae* pv. *oryzae* cells were the same as that of EdpX1-GFP. However, more than 92% of the signals of EdpX1^{ΔTM}-GFP diffused through the whole cells and did not show any specific foci. These results indicated that the TM domain is essential for the multisite localization of EdpX1, while the point mutations in the EAL domain do not affect its subcellular localization. To answer whether the localization was important for its regulatory function, we constructed the Δ *edpX1*(pB-*edpX1gfp*) and Δ *edpX1*(pB-*edpX1^{ΔTM}gfp*) strains and compared their ability to complement the virulence defect of the Δ *edpX1* mutant on rice leaves. The results showed that the two strains recovered the virulence to similar levels (Fig. 7), suggesting that the TM domain was not required for the EdpX1-mediated virulence regulation.

DISCUSSION

We have demonstrated that c-di-GMP signaling plays an important role in regulating the expression of different virulence factors in *X. oryzae* pv. *oryzae* (15, 39, 40). In this study, we characterized the function of the EAL domain protein EdpX1, which was demonstrated to be a PDE that positively regulates the virulence of *X. oryzae* pv. *oryzae*. We have previously shown that the GGDEF-EAL domain protein PdeR functions as a PDE and forms a two-component system with PdeK (15). Both PdeR and EdpX1 signaling pathways affected EPS production by controlling *gum* gene expression (Fig. 4) (15). Interestingly, neither PdeR nor EdpX1 seemed to play a role in regulating flagellar motility, which is one major output controlled by c-di-GMP. These observations suggest that the signaling pathways downstream of PdeR and EdpX1 may have similar

FIG 7 Transmembrane (TM) region was not required for the full function of EdpX1 to regulate the virulence of *X. oryzae* pv. *oryzae* on rice. (A) The following strains were inoculated on rice by leaf-clipping method: wild-type PXO99Δ(pBBR), the mutant ΔedpX1(pBBR) strain, and the complemented ΔedpX1(pB-edpX1gfp) and ΔedpX1(pB-edpX1ΔTMgfp) strains. The disease symptoms were observed on 14 dpi. (B) The lesion lengths were recorded. The error bar represents the standard deviation of the lesion lengths from 6 leaves. Three independent experiments were performed with similar results. Different letters (a to c) over the bars indicate a significant difference ($P < 0.05$) by the LSD test.

outputs, but their functions in regulating the virulence of *X. oryzae* pv. *oryzae* through c-di-GMP signaling are not redundant. Nevertheless, PdeR and EdpX1 are the major PDEs in *X. oryzae* pv. *oryzae* to modulate intracellular c-di-GMP levels and virulence-related phenotypes.

Structural and mutational analyses of EAL domains have proposed a two-metal-based mechanism for c-di-GMP hydrolysis by the EAL domain. The conserved residues participating in divalent metal cation (Mg^{2+} or Mn^{2+}) binding are necessary for hydrolysis of both c-di-GMP and bis-pNPP. In TBD1265 from *Thiobacillus denitrificans*, the E⁵²³ and E⁵⁴⁶ residues are involved in binding with Me and G1 substrates, respectively (37). The mutation of either E⁵²³ or E⁵⁴⁶ to A completely abolished the PDE activity of TBD1265. We generated point mutations of E¹⁵³ and E¹⁷⁶ on EdpX1, which correspond to E⁵²³ and E⁵⁴⁶ in TBD1265, respectively (Fig. 1). *In vivo* functional analysis and *in vitro* activity assays demonstrated that E¹⁵³, but not E¹⁷⁶, was essential for the PDE function of EdpX1 (Fig. 2), further supporting the theory that binding with metal ions is critical for the hydrolysis of c-di-GMP by EAL domains. The fact that EdpX1^{E153A} retained some activities in the *in vitro* assays might be due to the residual PDE activity from the purification procedure.

Mutation of *edpX1* in *X. oryzae* pv. *oryzae* caused defectiveness in both EPS production and biofilm formation. EPS secreted from xanthomonads is a polymer of repeating pentasaccharide units with a cellulose backbone and a trisaccharide side chain (46). A cluster of 12 genes (*gumB* to *gumM*) is responsible for the biogenesis of EPS (4, 47). The expression of representative *gum* genes, including *gumB*, *gumD*, and *gumK*, was reduced in the ΔedpX1 mutant and recovered to certain extents in the complemented strain. However, the genetic regulatory network that connects c-di-GMP signaling to *gum* gene expression needs further investigation.

Although it has been generally known that high c-di-GMP level promotes biofilm formation, there were also cases that did not conform to the principle (48, 49). For example, overexpression of two DGCs, PA2870 and PA3343 in *Pseudomonas aeruginosa*, did not alter the biofilm phenotype, although the c-di-GMP levels were boosted (49).

The deletion of the DGC gene *cgsA* in *Xylella fastidiosa*, which lowered the c-di-GMP level, promoted adhesiveness and biofilm formation (50). One of the reasons is probably because biofilm formation is a complex and dynamic process involving many factors, such as surface attachment, polysaccharide production, and flagellar motility (48, 50, 51). Individual DGC or PDE may control biofilm formation by specifically regulating one of these factors, which is largely determined by the functions of downstream c-di-GMP effectors (48, 51, 52). Considering that EPS is an essential component for the biofilm structure in *Xanthomonas* spp. (53), it was not surprising to observe decreased biofilm formation in the PDE mutant ΔedpX1 strain, which had significantly less EPS while no changes in motility. These results are consistent with the role of EdpX1 as a positive regulator for virulence in *X. oryzae* pv. *oryzae*. In contrast, the deletion of *dgcA*, which encodes a GGDEF domain protein confirmed to perform DGC activity, did result in decreased biofilm formation in *X. oryzae* pv. *oryzae*, despite the increased EPS production (54). This was probably due to the fact that the *dgcA* mutation had significantly enhanced the swimming motility of *X. oryzae* pv. *oryzae* (54). Nevertheless, it has becoming more and more difficult to generalize the connection between overall c-di-GMP level and the specific output for each PDE or DGC protein in *X. oryzae* pv. *oryzae*. Our results emphasized the importance of investigating in detail the function of each specific c-di-GMP enzyme.

We observed specialized subcellular localization of EdpX1 in the *X. oryzae* pv. *oryzae* cells. Its multisite localization was achieved via the TM domain, since EdpX1^{ΔTM}-GFP was evenly distributed through the whole cell (Fig. 6). It has been proposed that specific pools of c-di-GMP together with the effectors may facilitate temporal and spatial regulation of cellular functions (41, 55). Colocalization of an oxygen-sensing DGC/PDE, DocC/DocP, and the effector PNPase was observed in *Escherichia coli* (56). In other cases, global effectors, such as Clp or VpsT, may receive c-di-GMP signals from multiple enzymes (57, 58). Although the TM domain of EdpX1 seems not to be necessary for the EdpX1-mediated virulence regulation *in planta*, it is critical to identify the effector involved in the EdpX1 signaling pathway, which may further reveal the significance of the localization patterns of EdpX1.

MATERIALS AND METHODS

Bacterial strains and growth condition. The bacterial strains and plasmids used in this study are listed in Table 1. All primers are listed in Table 2. LB broth and LB agar were used for cultivating *Escherichia coli* at 37°C. The *X. oryzae* pv. *oryzae* wild-type PXO99^A and its derived strains were grown in M210 medium (0.8% casein enzymatic hydrolysate, 0.5% sucrose, 0.4% yeast extract, 17.2 mM K₂HPO₄, 1.2 mM MgSO₄·7H₂O [pH 7.0]) or peptone sucrose agar (PSA) medium with relevant antibiotics (59). Antibiotics were used at 100 μg/ml for ampicillin (Ap), 50 μg/ml for gentamicin (Gm), 50 μg/ml for kanamycin (Km), and 25 μg/ml for cephalixin (Cp).

Bioinformatic analysis. All sequences were downloaded from NCBI. The Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) was used to analyze the domains in EdpX1. Multiple-sequence alignments were carried out using the DNAMAN software (version 6.0; Lynnon BioSoft, Canada) and EMBL-EBI website servers (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

Construction of gene deletion mutant and complementation. The ΔedpX1 mutant derived from PXO99^A was constructed by homologous recombination, as described previously by using the suicide vector pK18mobSacB (60). The left and right arms of *edpX1* were ligated to pK18mobSacB, which carried a Gm resistance gene (14), resulting in plasmid pKedpX1G. The plasmid was introduced into the PXO99^A wild-type strain by electroporation. The potential mutants were screened as previously described (14). The single colonies that were resistant to Gm but sensitive to Km were considered mutant candidates.

The complemented strain was constructed as described below. The *edpX1* gene was amplified using the XD3877F and XD3877R primer pair. Then, it was digested and ligated with vector pBBR1MCS-4, resulting in plasmid pB-*edpX1*. pB-*edpX1* was then electroporated into the ΔedpX1 mutant, resulting in the ΔedpX1 (pB-*edpX1*) strain.

Determination of intracellular c-di-GMP levels. c-di-GMP levels in different strains were measured as previously described, with some modifications (61). Bacterial strains were cultured at 28°C in M210 overnight and then subcultured in 180 ml of fresh medium (1:100 dilution). The cultures were grown to mid-to-late-log phase when the optical density at 600 nm (OD₆₀₀) reached 2.5. The cell pellets were collected by centrifugation and resuspended in 1.5 ml of cold extraction buffer (40% methanol and 40% acetonitrile in 0.1 N formic acid) by pipetting. Then, the mixtures were incubated at –20°C overnight. After ultrasonication on ice for 1 min, the cultures were centrifuged at maximum speed for 20 min to pellet the cellular debris, and the debris-free liquid was collected as 400-μl aliquots and stored at –80°C. Before analysis, the frozen samples were dried by a vacuum freeze drier G25EL3 (Virtis, USA) and

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80(<i>lacZ</i>) Δ <i>lacX74</i> <i>hsdR</i> (r _K ⁻ m _K ⁺) Δ <i>recA1398</i> <i>endA1</i> <i>tonA</i>	TransGen Biotech, Beijing, China
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> PXO99 ^A	Wild-type strain, Philippine race, Cp ^r	Lab collection
Δ <i>edpX1</i> mutant	<i>edpX1</i> gene deletion mutant, Cp ^r Gm ^r	This work
Plasmid		
pK18mobsacB	Suicidal vector carrying <i>sacB</i> gene for mutagenesis, Km ^r	60
pKedpX1G	pK18mobsacB derivative for <i>edpX1</i> gene deletion, Km ^r Gm ^r	This work
pBBR1MCS-4	Broad-host-range expression vector, Ap ^r	70
pB- <i>edpX1</i>	pBBR1MCS-4 carrying full-length <i>edpX1</i> , Ap ^r	This work
pB- <i>edpX1</i> ^{E153A}	pBBR1MCS-4 carrying full-length <i>edpX1</i> with E153A point mutation, Ap ^r	This work
pB- <i>edpX1</i> ^{E176A}	pBBR1MCS-4 carrying full-length <i>edpX1</i> with E176A point mutation, Ap ^r	This work
pBGFP	pBBR1MCS-4 carrying fragment of GFP	39
pBedpX1gfp	pBGFP carrying full-length <i>edpX1</i> , Ap ^r	This work
pBedpX1 ^{E153A} gfp	pBGFP carrying full-length <i>edpX1</i> with E153A point mutation, Ap ^r	This work
pBedpX1 ^{E176A} gfp	pBGFP carrying full-length <i>edpX1</i> with E176A point mutation, Ap ^r	This work
pBedpX1 Δ TMgfp	pBGFP carrying EAL domain fragment of <i>edpX1</i> , Ap ^r	This work
pCold-SUMO	Protein expression vector, Ap ^r	Haigene
pCedpX1	pCold-SUMO carrying full-length <i>edpX1</i> , Ap ^r	This work
pCedpX1 ^{E153A}	pCold-SUMO carrying full-length <i>edpX1</i> with E153A point mutation, Ap ^r	This work
pCedpX1 ^{E176A}	pCold-SUMO carrying full-length <i>edpX1</i> with E176A point mutation, Ap ^r	This work

^aKm^r, kanamycin resistance; Ap^r, apramycin resistance; Gm^r, gentamicin resistance; Cp^r cephalixin resistance.

rehydrated with 100 μ l of high-performance liquid chromatography (HPLC)-grade water. Then, the samples were analyzed by using an Agilent 1290 liquid chromatograph equipped with a QQQ (Agilent6460, USA) tandem mass spectrometer. An Acquity ultraperformance liquid chromatography (UPLC) BEH C₁₈, 2.1 mm $\times$ 100 mm, 1.7 μ m column (Waters) was used for LC separation, using gradient elution with methanol solvent A and 10 mM tributylamine and 15 mM glacial acetic acid and water as solvent B. The c-di-GMP concentration of the sample was determined using a six-point standard curve of chemically synthesized c-di-GMP (Biolog) ranging from 0.5 to 25 ng/ml.

Site-directed mutagenesis. Mutations of E153A and E176A on the *edpX1* gene were generated by bridge PCR. For the mutation of E153, the upstream fragment was amplified using the primers XD3877F and XD3877P1-B, and the downstream fragment was amplified using the primers XD3877P1-C and XD3877R. The resulting fragments were mixed and used as the templates to amplify the fragment *edpX1*^{E153A} with primers PC3877F and PC3877R. The fragment was then inserted into protein expression vector pCold-SUMO (Haigene, China), resulting in plasmid pCedpX1^{E153A}. The mutation was confirmed by

TABLE 2 Primers used in this study

Primer name	Sequence (5'–3')	Purpose
XD3877F	CGGAATCTCGGCTTCGGATGTGTTAACAGGCCCTAGGAACGT	Δ <i>edpX1</i> complementation
XD3877R	CGAGCTCTCAGTGATGATGATGGTGATGATGCGCGCTGCTGC	Δ <i>edpX1</i> complementation
XD3877P1-B	CGGGTTGCTGCCAGCGCACCAGCGCTGCGG	<i>edpX1</i> ^{E153A} point mutation complementation
XD3877P1-C	CCTGCGTCGGCGCCGACGCGTGGT	<i>edpX1</i> ^{E153A} point mutation complementation
XD3877P2-B	CAGGATCAAGCCGCTTCTGCGGCC	<i>edpX1</i> ^{E176A} point mutation complementation
XD3877P2-C	TGCCTTCATTCCGCTGGCCGACAGAA	<i>edpX1</i> ^{E176A} point mutant complementation
PC3877F	CGGGATCCGTGTTAACAGGCCCTAGGAACGT	EdpX1 protein expression
PC3877R	GCGTCGACTCAATGCGCCGTGCTGC	EdpX1 protein expression
XDgF	CGGAATCCGTCGATGGGCTTCGGATGTGTTAACAGGCCCTAGGAACGT	EdpX1-GFP fluorescence microscopy assay
XDgR	CGGGATCCGTGATGATGATGGTGATGGCGCGCTGTGCGTGCTAG	EdpX1-GFP fluorescence microscopy assay
XDTMgF	CGGAATCCGTCGATGGGCTTCGGATGTGTTAACAGGCCCTAGGAACGT	EdpX1 Δ TM-GFP fluorescence microscopy assay
XDTMgR	CGGGATCCGTGATGATGATGGTGATGATGCGCCGTGCTGC	EdpX1 Δ TM-GFP fluorescence microscopy assay
gyrBF	GCGAGCACAAATGGCATT	qRT-PCR
gyrBR	CCATCCTTCTGCGGATGT	qRT-PCR
gumBF	GGCATGAGCGTTGGTGAG	qRT-PCR
gumBR	CCTGCTGGAGGGTCAGATT	qRT-PCR
gumDF	GCAGACAACACGCACTATGA	qRT-PCR
gumDR	AGCGACCAACGACGGATG	qRT-PCR
gumKF	ACGCCGAGACGATTGGTT	qRT-PCR
gumK-R	CCACCGCATTCGGACATAC	qRT-PCR

sequencing. pCedpX1^{E176A} was constructed similarly. For the complementation experiments, the DNA fragments of edpX1^{E153A} and edpX1^{E176A} were amplified by primer pair XD3877F and XD3877R using pCedpX1^{E153A} and pCedpX1^{E176A} as the templates, respectively. The fragments were inserted into broad-host-vector pBBR1MCS-4, resulting in pB-edpX1^{E153A} and pB-edpX1^{E176A}.

Protein expression and purification. pCold-SUMO-derived constructs were transformed into *E. coli* BL21 strains for protein expression and purification. Protein expression was induced with 0.1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for 24 h at 15°C. Subsequently, purification of target proteins was performed according to methods described previously (15).

Phosphodiesterase colorimetric assay. *In vitro* purified proteins were assayed for the PDE activity using colorimetric assays (15, 32). Briefly, purified proteins (20 μ g) were incubated with 5 mM bis-pNPP at 37°C for 2 h in assay buffer (50 mM Tris-HCl, 1 mM MnCl₂ [pH 8.5]). The yellow product was quantified using a spectrophotometer at OD₄₁₀. Bovine serum albumin (BSA) was used as a negative control. The experiment was repeated three times using three batches of independently purified proteins.

Virulence assay. The virulence of *X. oryzae* pv. *oryzae* was tested on the susceptible rice plant *Oryza sativa* subsp. *japonica*. Test strains were grown in M210 with appropriate antibiotics until the OD₆₀₀ reached 0.8. The cells were collected by centrifugation and resuspended in the same volume of sterile distilled water. The bacterial cells were inoculated onto the leaves of 6-week-old rice plants using the leaf-clipping method (8). Then, disease lesion lengths were measured on 14 dpi. The experiments were repeated three independent times.

RNA extraction and quantitative real-time PCR assay. Bacterial cells were grown in M210 at 28°C until the OD₆₀₀ reached 1.0 and were harvested by centrifugation. Total RNAs were extracted using the RNeasy pure cell/bacteria kit (Qiagen, Beijing, China), following the manufacturer's instructions. HiScript II Q RT SuperMix for qPCR (Vazyme Biotech) was used to synthesize cDNA. Quantitative real-time RT-PCR (qRT-PCR) was performed using 2 ng cDNA and AceQ quantitative PCR (qPCR) SYBR green master mix (low ROX premixed; Vazyme Biotech, Nanjing, China) on an Applied Biosystems 7500 real-time PCR system. The relative levels of gene expression were determined by using the threshold cycle $2^{-\Delta\Delta CT}$ method (62) with the DNA gyrase subunit B gene (*gyrB*) as the internal control (63). Three technical replicates were used each time.

EPS assay. Bacterial strains were grown in M210 medium until the OD₆₀₀ reached 2.5, and the supernatants were collected by centrifugation at 8,000 rpm for 10 min. Two volumes of ethanol were added to the supernatants, and the resulting solution was incubated at -20°C overnight. The precipitated EPS was collected by centrifugation at 8,000 rpm for 20 min and dried overnight at 55°C, and dry weights were recorded (64). For the plate assay, strains were grown in M210 until the OD₆₀₀ reached 0.8. Two microliters of culture was spotted onto a PSA plate without any antibiotics and incubated at 28°C for 4 days. Then, photos were taken for the colonies. The experiments were independently repeated three times.

Biofilm formation assay. Log-phase-grown bacteria were diluted in M210 to an OD₆₀₀ of 0.5 and incubated at 28°C for 4 days in the 96-well culture plate. Bacterial pellicles were stained by gently pouring off the medium, washing once with water, and staining with 0.1% crystal violet. Cells were washed and rinsed with water until all unbound dye was removed. The adherent dye was later redissolved with absolute ethanol and quantified by spectrophotometry at OD₄₉₀ (65).

Fluorescence microscopy. Plasmids pBedpX1gfp, pBedpX1^{E153A}gfp, pBedpX1^{E176A}gfp, and pBedpX1 Δ TMgfp were constructed first. For pBedpX1gfp, pBedpX1^{E153A}gfp, and pBedpX1^{E176A}gfp, primers XDgF and XDgR were used for amplifying the target fragments using pB-edpX1, pBedpX1^{E153A}, and pBedpX1^{E176A}, respectively, as the templates. For pBedpX1 Δ TMgfp, primers XDTMgF and XDTMgR were used to amplify the target fragment, with pB-edpX1 as the template. Then, the fragment was then inserted into pBGFP (39). The plasmids were electroporated into PXO99^A, resulting in strains PXO99^A(pBedpX1gfp), PXO99^A(pBedpX1^{E153A}gfp), PXO99^A(pBedpX1^{E176A}gfp), and PXO99^A(pBedpX1 Δ TMgfp). The strains were cultivated in M210 at 28°C until the OD₆₀₀ reached 1.0. Then, the bacterial cells were dropped on the glass slide. Subcellular signals of each GFP fusion were observed and recorded using the Olympus BX61 fluorescence microscope (Japan).

Statistical analysis. The means and standard deviations of experimental results were calculated using Excel. Fisher's least significant difference (LSD) test was performed using the DPS data processing system (http://www.dpsw.cn/dps_eng).

Phylogenetic analysis. Seventeen homologous proteins of EdpX1 from different *Xanthomonas* spp. were selected for phylogenetic analysis. Amino acid sequence alignment was performed using DNAMAN and MEGA7 ClustalW (66). The protein weight matrix used GONNET, and other parameters were default values. The phylogenetic tree was constructed using MEGA7. The evolutionary history was inferred using the neighbor-joining method (67). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) were shown next to the branches (68). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated using the *p*-distance method (69).

Western blotting. The samples were boiled and separated in SDS gels containing 12% acrylamide and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore). After 2 h of saturation at room temperature in phosphate-buffered saline (PBS), 0.1% Tween 20, and 10% skimmed milk, the membrane was incubated overnight at 4°C in PBS-0.1% Tween 20 and 10% skimmed milk with appropriated antibodies (1:5,000 for anti-His) (Huaxingbio, China), washed three times with PBS-0.1% Tween 20, incubated for 1 h in PBS-0.1% Tween 20 and 10% skimmed milk with anti-mouse conjugate horseradish

peroxidase (HRP) antibody (Sigma) (1:10,000), washed three times with PBS–0.1% Tween 20, and then revealed with TYPhoon9410 (Amersham, USA).

Swimming assay. Bacterial strains were grown in M210 medium until the OD₆₀₀ reached 0.8. Two microliters of culture was spotted onto semisolid plates containing 0.03% Bacto peptone, 0.03% yeast extract, and 0.25% agar. Plates were incubated at 28°C for 4 days. The diameters of the motility zones indicated bacterial movement ability. The experiment was repeated at least three times with three replicates for each time.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01717-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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