Xanthomonas oryzae pv. *oryzae* Response Regulator TriP Regulates Virulence and Exopolysaccharide Production Via Interacting With c-di-GMP Phosphodiesterase PdeR

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PdeR, a response regulator of the two-component system (TCS) with the cognate histidine kinase PdeK, has been shown to be an active phosphodiesterase (PDE) for intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) turnover and positively regulates the virulence of *Xanthomonas oryzae* pv. *oryzae*, the causal pathogen of bacterial blight of rice. To further reveal the key components and pathways involved in the PdeR-mediated c-di-GMP regulation of virulence, 16 PdeR-interacting proteins were identified, using the yeast two-hybrid (Y2H) assay. Among them, PXO_04421 (named as TriP, a putative transcriptional regulator interacting with PdeR) was verified via Y2H and glutathione-S-transferase pull-down assays, and its regulatory functions in bacterial virulence and exopolysaccharide (EPS) production were assessed by biochemical and genetic analysis. The REC domain of TriP specifically interacted with the EAL domain of PdeR. TriP promoted the PDE activity of PdeR to degrade c-di-GMP in the presence of PdeK. In-frame deletion in *triP* abolished the polar localization of PdeR in the cell. Notably, the $\Delta triP$ mutant showed significantly reduced virulence on susceptible rice leaves and impaired EPS production compared with wild type, whereas the double mutant $\Delta triP \Delta pdeR$, like $\Delta pdeR$, caused shorter lesion lengths and produced less EPS than $\Delta triP$. In addition, cross-complementation showed in *trans* expression of *pdeR* in $\Delta triP$ restored its EPS production to near wild-type levels but not vice versa. Taken together, our results suggest

that TriP is a novel regulator that is epistatic to PdeR in positively regulating virulence expression in *X. oryzae* pv. *oryzae*.

Two-component systems (TCSs) consisting of histidine kinases (HKs) and response regulators (RRs) are predominant for signal transduction in bacteria (Jung et al. 2012; Stock et al. 2000; Zschiedrich et al. 2016). TCSs help bacteria to sense, respond, and adapt to changes in their surroundings, including environmental and host conditions. Usually, the signal transduction involves autophosphorylation at the histidine residue of a HK upon sensing certain signals and, then, transfer of the phosphoryl group to a conserved aspartate residue on the receiver domain of its cognate RR. Although the majority of RRs function as transcriptional regulators that trigger responses by directly affecting gene expression, there are other types of signaling outputs related to enzymatic regulation or protein-protein interactions (Gao and Stock 2009; Jung et al. 2012; Skerker et al. 2005).

Bacterial second messengers are intracellular signaling molecules that trigger physiological changes. Cyclic dimeric guanosine monophosphate (c-di-GMP), a nucleotide-type second messenger, has been shown to regulate the bacterium life-style transition from single planktonic cells to a sessile, multicellular biofilm (Povolotsky and Hengge 2012; Römling et al. 2013; Simm et al. 2004). It has also been implicated in regulating other cellular behaviors such as cell-cycle progression, flagellar motility, and virulence (Abel et al. 2011; Fang and Gommelsky 2010; Paul et al. 2010; Yang et al. 2012; Yuan et al. 2015). Extensive structural and functional studies have revealed that synthesis and degradation of c-di-GMP is performed by GGDEF domain-containing diguanylate cyclases (DGCs) and EAL- or HD-GYP domain-containing phosphodiesterases (PDEs), respectively (Schirmer and Jenal 2009). c-di-GMP effectors, which are capable of binding to c-di-GMP, can sense intracellular c-di-GMP levels and trigger physiological changes accordingly (Sondermann et al. 2012). So far, several different types of c-di-GMP effectors have been identified, including PilZ-domains, various transcriptional factors, degenerated GGDEF/EAL domains, riboswitches, and recently defined ATPases of secretion systems (Jones et al. 2015; Roelofs et al. 2015; Sondermann et al. 2012).

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight of rice, which is the most severe bacterial disease of this staple

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crop worldwide. *X. oryzae* pv. *oryzae* enters rice leaves through wounds or water pores, leading to systemic infection in the xylem tissue of susceptible rice cultivars (Niño-Liu et al. 2006). Whether or not the disease occurs is largely dependent on the interactions between the type III effectors and their target genes in the host (White and Yang 2009; Zhang and Wang 2013). Besides the type III secretion system, virulence factors such as exopolysaccharides (EPS), extracellular enzymes, and the type II secretion system are also critical for the pathogenesis of *X. oryzae* pv. *oryzae* (Ray et al. 2000; Sun et al. 2005; Zhou et al. 2013).

In the past few years, we have uncovered several components in the c-di-GMP signaling pathway of *X. oryzae* pv. *oryzae*. PdeR is an active PDE and a TCS RR and, together with its cognate HK PdeK, positively regulates the EPS production and virulence of *X. oryzae* pv. *oryzae* (Yang et al. 2012). Similar to PdeR, EdpX1 functions as a PDE to degrade c-di-GMP and promotes EPS production, biofilm formation, and virulence (Xue et al. 2018). A putative DGC, GdpX1, controls EPS production and virulence in a manner opposite to PdeR (Yang

et al. 2016). This regulation scheme is consistent with the general theory that low c-di-GMP promotes virulence, while high c-di-GMP inhibits virulence. In addition, several different types of c-di-GMP receptors and effectors have been identified from *X. oryzae* pv. *oryzae*, including the degenerated GGDEF/EAL domain protein Filp, the PilZ domain proteins PXO_00049, PXO_02374, and PXO_02715, and the transcriptional regulator Clp (Li et al. 2013; Yang et al. 2014, 2015).

In this study, we focused on a complicated PdeR-dependent signaling pathway that regulates a plethora of virulence factors in *X. oryzae* pv. *oryzae*. We identified PXO_04421 as an interactor of PdeR, which was named as TriP (a putative transcriptional regulator interacting with PdeR). Their genetic and biochemical relationship was also investigated, and the results showed TriP might be epistatic to PdeR in regulating virulence-related traits of *X. oryzae* pv. *oryzae*.

RESULTS

The response regulator TriP specifically interacts with the phosphodiesterase PdeR.

To uncover the PdeR-mediated c-di-GMP signaling pathway and its regulation of virulence in *X. oryzae* pv. *oryzae*, we started by looking for proteins that interact with PdeR in *X. oryzae* pv. *oryzae*. Yeast two-hybrid (Y2H) assays were performed using PdeR as a bait expressed in the Y2HGold strain against the prey library of *X. oryzae* pv. *oryzae* constructed in the host strain Y187. A total of 16 putative PdeR-interacting proteins were found after the screening process (Supplementary Table S1). Among them, PXO_04421 was annotated as an OmpR-family TCS response regulator. The full length of PXO_04421 consisted of an N terminal REC domain (6 to 117 aa) and a C terminal helix-turn-helix (HTH) DNA-binding domain (157 to 232 aa). Thus, we named it TriP. To test whether TriP is a bona fide interactor of PdeR, we cloned the full length of the *triP* gene into prey vector pGADT7 and transformed it into the Y187 strain. Interaction of AD-TriP with DBD-PdeR or DBD-PdeK was directly tested, using Y2H assays (Fig. 1A). Yeast zygotes containing AD-TriP and DBD-PdeR showed robust growth on quadruple drop out (QDO) plates in 2 days, suggesting their interaction was strong. In contrast, no interaction between TriP and PdeK was observed. In addition, glutathione-S-transferase (GST) pull-down assays were carried out to further confirm the interaction between PdeR and TriP. GST-TriP, GST tag only, and PdeR-His₆ were expressed and purified. Different combinations of the purified proteins were mixed and were then precipitated with GST binding beads. The coeluted proteins were detected by Western blotting, using anti-GST or anti-His antibodies. PdeR-His₆ was the only protein detected when GST-TriP was present in the mixture (Fig. 1B), indicating that TriP could pull PdeR down. Moreover, no interaction between TriP and PdeK was observed (Fig. 1A). These results provide evidence that TriP physically and specifically interacts with PdeR.

The TriP REC domain specifically interacts with the PdeR EAL domain.

Since both PdeR and TriP are multidomain proteins consisting of the GGDEF-EAL-REC and REC-HTH domains, respectively, we questioned which domains are essential for their interaction. Therefore, Y2H assays were employed to determine the interaction between structural variations of proteins PdeR and TriP. The N-terminal half of TriP (TriP-N) and its C-terminal half (TriP-C) were cloned into the pGADT7 vectors, and their interactions with DBD-PdeR were tested. The results showed that TriP-N but not TriP-C was able to interact with the

Fig. 1. Interaction between PdeR and TriP was confirmed by yeast two-hybrid (Y2H) assay and glutathione-S-transferase (GST) pull-down assay. **A**, Interaction between full-length PdeR and TriP was detected in the Y2H assay. The Y2HGold yeast strains carrying pGBKPdeR and Y187 yeast strains carrying pGADTriP were mated and selected on double drop-out (DDO) medium and were further examined on quadruple drop-out (QDO) medium. Serial dilutions (10-fold) were performed to evaluate the interaction strength. Pictures were taken after the cells grew for 2 days. **B**, Interaction between PdeR and TriP was confirmed by the GST pull-down assay. Purified PdeR-His₆ fusion protein was incubated with an equal amount of purified GST-TriP and were precipitated by the GST binding beads. The coeluted proteins were detected by Western blotting using anti-GST (lower panel) or anti-His (upper panel) antibodies.

full-length PdeR (Fig. 2A). Then, we examined which domain of PdeR was essential for its interaction with TriP-N. Each domain of PdeR, including GGDEF(G), EAL(E), and REC(R), were cloned into the pGBKT7 vector. However, we were not able to get the Y2HGold transformants for pGBKT7 carrying PdeR-E (pGBKPdeR-E). Therefore, the interaction between PdeR-E and TriP-N was tested after switching the vectors for these two domains. The results showed that the EAL domain of PdeR specifically interacted with TriP-N (Fig. 2A). These observations suggest that the REC domain of TriP and the EAL domain of PdeR are essential for the two-protein interaction (Fig. 2B).

TriP enhances the PDE activity of PdeR for c-di-GMP turnover.

Since PdeR harbors PDE activity to degrade c-di-GMP and TriP is identified as a novel interactor of PdeR, it is important to know whether TriP affects the enzymatic activity of PdeR. To test this, colorimetric assays were carried out to examine PDE activities in different protein combinations. GST tag alone or recombinant proteins GST-TriP, PdeR-His₆, and PdeK-His₆ were purified to homogeneity (Fig. 3A). As expected, PdeR alone did not show any PDE activity. With the addition of ATP and its cognate HK PdeK, the PDE activity of PdeR was significantly increased (Fig. 3B). TriP, which does not harbor any PDE-encoding domain, did not exhibit any c-di-GMP degrading ability. As shown in Figure 3, TriP itself was able to trigger PdeR PDE activity in the absence of PdeK, although the activity was relatively lower. PDE activity of PdeR was enhanced after addition of TriP into the ATP+PdeK+PdeR mixture in a concentration-dependent manner (Fig. 3B), which was an unexpected observation. This experimental data demonstrates that TriP promotes the PDE activity of PdeR in the presence of PdeK.

To provide additional evidence that intracellular c-di-GMP levels are affected by TriP, the c-di-GMP contents in wild type, $\Delta triP$, $\Delta triP$ (pBTriP), $\Delta pdeR$, $\Delta pdeK$, and $\Delta triP\Delta pdeR$ were examined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Like $\Delta pdeR$ and $\Delta pdeK$, $\Delta triP$ showed significant increases in c-di-GMP concentration compared with wild type (Fig. 4). In addition, the elevated c-di-GMP amount was restored to the wild-type level in complementary strain $\Delta triP$ (pBTriP). In addition, $\Delta triP\Delta pdeR$ displayed a higher c-di-GMP level than $\Delta triP$. These findings demonstrate that TriP provides a significant negative influence over intracellular levels of c-di-GMP.

TriP affects polar localization of PdeR in *X. oryzae* pv. *oryzae* cells.

Subcellular compartmentation of c-di-GMP signaling components has been suggested to facilitate temporal and spatial regulation of specific activities (Güvener and Harwood 2007; Merritt et al. 2010; Paul et al. 2008). PdeR has been demonstrated to physically interact with both its cognate HK PdeK (Yang et al. 2012) and a transcriptional regulator, TriP, in this study. We will next address whether these proteins have specific localization patterns and whether colocalization occurs between them. To test this, C-terminal green fluorescent protein (GFP) fusions of TriP, PdeR, and PdeK were generated and were observed for their subcellular localization in *X. oryzae* pv. *oryzae* under fluorescence microscopy. As shown in Figure 5, PdeR demonstrated bipolar localization in most cells. Comparatively, PdeK was not only localized to the poles but, also, had multiple foci in most cells. It is possible that PdeK might function as a putative HK to sense environmental signals. When both PdeK and PdeR are localized to the poles, it might help to increase the rate of phosphorylation from PdeK to PdeR, thus

increasing the concurrent PDE activity of PdeR. Different from PdeR and PdeK, which showed specific foci, the signal of TriP-GFP diffused throughout the whole cell. However, it is still possible that transient interaction between TriP and PdeR occurs in the cytoplasm.

Since both PdeK and TriP seem to be epistatic to PdeR in the regulatory pathway, we next asked whether PdeK or TriP influences the subcellular localization of PdeR in *X. oryzae* pv. *oryzae*. We examined the localization patterns of PdeR-GFP in the $\Delta pdeK$ and $\Delta triP$ mutants (Fig. 5). Interestingly, PdeR retained its bipolar localization in $\Delta pdeK$, the same as the wild-type strain, indicating no effect of PdeK on PdeR localization. However, the bipolar localization pattern disappeared in $\Delta triP$, suggesting TriP significantly influenced the subcellular localization of PdeR. These results obviously indicate the necessity of TriP but not PdeK for bipolar localization of PdeR in *X. oryzae* pv. *oryzae*.

TriP is required for optimal virulence of *X. oryzae* pv. *oryzae* in rice.

The observation that TriP influences the PDE activity of PdeR prompted us to examine whether TriP also regulates the virulence of *X. oryzae* pv. *oryzae*. An in-frame deletion mutant

Fig. 2. Determination of specific domains required for the interaction between PdeR and TriP. **A**, Interactions between different fragments of PdeR and TriP were tested in yeast two-hybrid (Y2H) assays. Different combinations were detected between Y187 yeast strains carrying pGADT7-derived constructs to express the N terminus of TriP (TriP-N), the C terminus of TriP (TriP-C), and the EAL domain of PdeR (PdeR-E). Y2HGold yeast strains carrying pGBKT7-derived constructs were used to express PdeR, the GGDEF domain of PdeR (PdeR-G), the REC domain of PdeR (PdeR-R) and TriP-N, respectively. **B**, Interaction between different domains are schematically summarized. The REC domain of TriP and the EAL domain of PdeR were required for the interaction. Numbers represent amino acid residues where the predicted domains start and end, based on the National Center for Biotechnology Information conserved domain database and the SMART database.

of *triP* was tested for its virulence on a susceptible rice variety (*Oryza sativa* L cv. Nipponbare), using the leaf-clipping method. The disease symptoms and the lesion lengths were scored 14 days postinoculation. Compared with the wild-type strain, disease severity caused by the $\Delta triP$ mutant was decreased, resulting in shorter lesion lengths, and the complemented strain containing a plasmid to express full-length *triP* in *trans* could restore the diseased phenotypes to near-wild-type levels (Fig. 6). To further characterize the genetic relationship between TriP and PdeR, we constructed a $\Delta triP\Delta pdeR$ double mutant and tested its virulence. The $\Delta triP\Delta pdeR$ double mutant caused similar disease symptoms and lesion lengths to the $\Delta pdeR$ mutant, which was less severe than the $\Delta triP$ mutant (Fig. 6). Moreover, to clarify whether the reduction in virulence of the mutants is due to reduction in growth, growth rates of the wild type, $\Delta triP$, $\Delta pdeR$, and $\Delta triP\Delta pdeR$ strains were measured in M210 liquid medium. No significant differences were observed in growth rates between the four strains (Supplementary Fig. S1), suggesting that gene deletion of either *triP*, *pdeR*, or both does not significantly affect the viability of *X. oryzae* pv. *oryzae* under normal growth conditions. Accordingly, these findings reveal that TriP and PdeR are both required for the virulence of *X. oryzae* pv. *oryzae* to host plants and TriP might act upstream of PdeR in the regulatory pathway of virulence in *X. oryzae* pv. *oryzae*.

TriP is epistatic to PdeR in regulating EPS production.

Since EPS is generally regarded as an important virulence factor for *X. oryzae* pv. *oryzae* and reduced virulence on rice is displayed in $\Delta triP$, we tested whether its EPS production was affected. An approximately 10% decrease in EPS secretion of $\Delta triP$ was observed, compared with that of wild type, while the complemented strain restored EPS production to near wild-type levels (Fig. 7). In contrast to $\Delta triP$, a 25% reduction of EPS was observed in $\Delta pdeR$. In addition, EPS production was examined in the $\Delta triP\Delta pdeR$ double mutant, and interestingly, it was reduced to a level that was very similar to that of $\Delta pdeR$. This was consistent with the virulence phenotypes observed previously (Fig. 6). To further investigate their genetic relationship, we constructed cross-complementation strains $\Delta pdeR(pBTriP)$ and

$\Delta triP(pBPdeR)$ and examined their EPS production levels. EPS secreted from $\Delta triP(pBPdeR)$ was restored to wild-type level, while that from $\Delta pdeR(pBTriP)$ could not be restored (Fig. 7). These observations reveal that TriP might be epistatic to PdeR in positively regulating EPS production of *X. oryzae* pv. *oryzae*.

DISCUSSION

Resolving the highly complex c-di-GMP signaling pathway is of utmost importance for a comprehensive understanding of molecular mechanisms underlying virulence expression and

Fig. 4. Measurement of intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) levels of *Xanthomonas oryzae* pv. *oryzae* strains. The intracellular c-di-GMP concentrations in wild-type PXO99^A, $\Delta triP$, $\Delta triP(pBTriP)$, $\Delta pdeR$, $\Delta pdeK$, and $\Delta triP\Delta pdeR$ were assayed by liquid chromatography-tandem mass spectrometry. Error bars represent standard deviation from three biological repeats. Lower case letters (a, b, and c) indicate significant difference ($P < 0.05$) by Fisher's least significant difference test.

Fig. 3. Colorimetric assay to detect phosphodiesterase (PDE) activity of PdeR. **A**, The purified proteins of glutathione-S-transferase (GST), GST-TriP, PdeR-His₆, and PdeK-His₆ were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. **B**, Bis (p-nitrophenyl) phosphate substrate was used for the PDE colorimetric reaction, and the hydrolysis of the substrate was detected spectrophotometrically at a wavelength of 410 nm. The error bars represent standard deviations in triplicate.

regulation in pathogenic bacteria (Römling et al. 2013). We have previously demonstrated that PdeR, an active PDE for c-di-GMP turnover, positively regulates virulence expression in *X. oryzae* pv. *oryzae* (Yang et al. 2012). To further reveal key components and pathways of PdeR-mediated c-di-GMP signaling involved in regulation of virulence, we identified and characterized TriP, a novel RR that interacted with PdeR in this study. TriP was demonstrated to enhance PDE activity for c-di-GMP hydrolysis and proper intercellular bipolar localization of PdeR. In addition, TriP was shown to positively influence bacterial virulence on rice as well as EPS production. To our knowledge, this is the first study that shows that TriP functions as a novel key regulator of virulence expression via a PdeR-mediated c-di-GMP signaling pathway.

The identification of the TriP-PdeR regulatory cascade prompts us to speculate about possible interaction patterns of two TCS RRs in *X. oryzae* pv. *oryzae*. It has been generally recognized that HKs detect and translate input information and RRs control the output during the TCS-mediated signal transduction pathway (Jung et al. 2012; Zschiedrich et al. 2016). In RRs, REC domains typically function as

phosphorylation-mediated switches, catalyze their own phosphorylation and dephosphorylation, and activate the output domains, such as HTH DNA-binding or EAL and GGDEF domains (Bourret 2010; Gao and Stock 2009). Based on bioinformatic analysis of TriP that possessed both REC and HTH domains (Fig. 2B), we defined TriP as a ‘characteristic’ RR. TriP was shown to interact directly with TriK, an OmpR family TCS sensor kinase (Supplementary Fig. S2), indicating TriK/TriP might be a novel pair of TCS in *X. oryzae* pv. *oryzae*. More interestingly, PdeR, consisting of the GGDEF-EAL-REC domains, forms a cognate pair with the HK PdeK to regulate bacterial virulence (Yang et al. 2012). Here, we revealed that the TriP REC domain had a novel and additional function in specific binding to the PdeR EAL domain (Fig. 2A), while TriP didn’t interact with PdeK (Fig. 1A). We also believe that this is the first report to identify the REC-EAL domain interaction of two RRs, while also illustrating the function of TriP as a PDE interactor in *X. oryzae* pv. *oryzae*.

Since degradation of c-di-GMP is catalyzed by PDEs, regulation of PDE hydrolytic activity is crucial for modulation of cellular functions (Römling et al. 2017). It has been shown that

Fig. 5. Subcellular localization of green fluorescent protein (GFP) fusion proteins in *Xanthomonas oryzae* pv. *oryzae* strains. *X. oryzae* pv. *oryzae* strains were cultured in M210 medium at 28°C, until the optical density at 600 nm = 0.6, were diluted and 3 μ l of cell suspension was spotted on a glass slide coated with 1% agarose. Photographs of GFP fusion proteins were taken using a fluorescence microscope (Olympus BX61), and subcellular localization was counted in at least 10 fields of view. The scale bar represents 2 μ m in length. Numbers indicate different locations: 1, unipolar; 2, bipolar; 3, multisite; and 4, cytoplasmic.

local c-di-GMP signaling relies on direct protein-protein interactions of one or both a PDE and a DGC with a specific c-di-GMP-binding (Dahlstrom et al. 2015; Lindenberg et al. 2013). Such a protein interaction can establish a local 'source' and 'sink' of c-di-GMP in the immediate vicinity of a c-di-GMP-controlled machinery (Hengge 2016). Though more remains to be elucidated regarding the relevant regulatory interaction of TriP with PdeR, the plausible mechanism underlying the physical domain interaction of the two RRs can be postulated. We provide four possible hypotheses: i) allosteric regulation of PdeR may occur upon interacting with TriP in the presence of the substrate c-di-GMP, thereby leading to enhanced enzyme activity of PdeR; ii) TriP regulation of

bipolar localization of PdeR may be at the level of protein-protein interaction but not transcription, since TriP did not affect the transcription of either *pdeR* or *pdeK*, as revealed by our unpublished RNA-seq analysis data; iii) the phenomenon that TriP deletion results in significant increases of intracellular c-di-GMP might be due to an influence on the function of TriP itself as a putative response regulator; and iv) TriP itself can activate PdeR PDE activity without PdeK and no direct interaction exists between TriP and PdeK, suggesting TriP might not act as an intermediary between PdeK and PdeR or as a phosphor donor itself. PdeR might be activated independently by both PdeK phosphorylation and TriP interaction. Preliminary bioinformatic analysis of the TriP-PdeR interaction predicted the REC and EAL domain interface structures (Supplementary Fig. S3). To further understand the exact molecular basis underlying this regulatory cascade, providing the crystal structure defining the protein-protein interaction of TriP and PdeR may provide some insights.

We found that TriP was necessary for full virulence in rice and EPS production, one of the most critical pathogenicity factors in *X. oryzae* pv. *oryzae*. Δ TriP was shown to significantly reduce virulence on rice and impaired EPS production compared with the wild type, whereas the double mutant Δ TriP Δ pdeR, like Δ pdeR, caused shorter bacterial blight lesion lengths and produced less EPS than Δ TriP (Figs. 6 and 7). In *trans* complementation of *pdeR* in Δ TriP restored its virulence and EPS production to near wild-type levels but not vice versa (Fig. 7). Combined with our previous report that both Δ pdeK and Δ pdeR showed decreased virulence on rice but the virulence of Δ pdeR was more significantly affected (Yang et al. 2012), we believe that Δ TriP was less affected in virulence than Δ pdeR. Thus, we conclude that TriP functions upstream of PdeR in the TriP-PdeR regulatory cascade in *X. oryzae* pv. *oryzae*.

The impact of TriP on bacterial virulence and EPS production was less than that of PdeR alone, as discussed above; however, Δ TriP showed intracellular c-di-GMP levels similar to

Fig. 6. Virulence test of *Xanthomonas oryzae* pv. *oryzae* strains on susceptible rice variety (*Oryza sativa* L. cv. Nipponbare). Virulence of *X. oryzae* pv. *oryzae* strains was assessed by the leaf-clipping method. **A**, Symptoms were recorded by photography and **B**, the lesion lengths were measured for individual inoculated leaves 14 days after inoculation. Values are means and error bars represent standard deviations from 20 inoculated leaves in one experiment. The experiment was repeated four times with similar results. Lower case letters (a, b, and c) indicate significant difference ($P < 0.05$) by Fisher's least significant difference test.

Fig. 7. Quantitative and phenotypic assays for exopolysaccharide (EPS) production of *Xanthomonas oryzae* pv. *oryzae* strains. EPS quantitative assays of Δ TriP, Δ TriP(pBTriP), Δ pdeR, Δ TriP Δ pdeR, Δ pdeR(pBTriP), Δ TriP(pBPdeR-GFP), and PXO99^Δ(pBPdeR-GFP) strains were determined by the weight method, compared with the level of wild-type PXO99^Δ. Phenotypes were recorded by a Canon 550D digital camera. Values are means and error bars represent standard deviation from three repeats. Lower case letters (a, b, and c) indicate significant difference ($P < 0.05$) by Fisher's least significant difference test.

that of *ΔpdeR* (Fig. 4). We speculate that TriP may function in the regulation of bacterial virulence phenotypes on many levels. For example, TriP regulates virulence and EPS production by interacting with PdeR at the protein-protein level, as revealed in this work. A working model outlining TriP regulation of virulence expression via interacting with PdeR in *X. oryzae* pv. *oryzae* has been proposed (Fig. 8). In addition, TriP may also mediate its impact on virulence expression at the transcriptional level. TriP, a putative transcriptional regulator, may target a set of genes encoding the downstream key components in c-di-GMP signaling pathways, thereby modulating virulence expression in the pathogen. Further RNA-seq analysis of TriP will identify these important components and their roles in the TriP-mediated regulation of virulence in *X. oryzae* pv. *oryzae*.

Taken together, the identification of the novel regulator TriP will increase our current understanding of the interplay and functionality of RRs, which are involved in the c-di-GMP signaling network in pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers.

Escherichia coli strains were grown at 37°C in Luria Bertani (LB) medium (Hanahan 1983). *X. oryzae* pv. *oryzae* wild-type strain PXO99^A (Hopkins et al. 1992) and derived mutant strains were cultured at 28°C in M210 medium (0.8% casein enzymatic hydrolysate, 0.5% sucrose, 0.4% yeast extract, 0.03% MgSO₄·7H₂O, pH 7.0) or peptone sucrose agar (PSA) (1% peptone, 1% sucrose, 1% glutamic acid, 1.5% agar, pH 7.0) medium with relevant antibiotics. The antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; gentamycin 50 μg/ml; and cephalexin 20 μg/ml. Bacterial strains, plasmids, and primers used in this study are listed in Table 1.

RNA isolation and cDNA library construction.

To extract RNA from PXO99^A, bacterial strains were grown in M210 at 28°C till it reached an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8. A bacterial RNAout (Tiangen Biotech Co., Beijing) kit was used for total RNA isolation according to the manufacturer instructions. Total RNA was treated with DNase I (Tiangen Biotech Co.) to remove DNA contamination. The cDNA library for Y2H screening was generated using the Make Your Own “Mate & Plate” Library System (Clontech Laboratories, Mountain View, CA, U.S.A.) according to the manual description. In brief, 5 μg of total RNA served as the template for cDNA synthesis utilizing the SMARTIII technology. Double-stranded cDNA less than 400 bp were discarded, using Chroma Spin TE-400 columns, followed by cotransformation with pGADT7 into competent yeast strain Y187. Then, pelleted cells were resuspended in 15 ml of 0.9% (wt/vol) NaCl solution and spread on selection agar plates depleting leucine (SD/-Leu). Transformants were pooled and kept in 1-ml aliquots and were stored at -80°C until use.

Y2H screening and assay.

For Y2H screening, a 1-ml library aliquot of *X. oryzae* pv. *oryzae* cDNA library was used as prey, and Y2HGold containing pGBKPdeR was used as the bait strain (Yang et al. 2012). They were mixed and incubated at 30°C for 24 h. The mixture was centrifuged and the resuspended pellets were spread onto stringency SD/-Leu/-Trp plates containing aureobasidin A and X-α-gal (double drop out [DDO/X/A]), followed by higher stringency SD/-Ade/-His/-Leu/-Trp supplemented with aureobasidin A and X-α-gal (QDO/X/A). Total surviving blue colonies on QDO were subjected to polymerase chain

reaction (PCR) and sequencing. Sequences were compared with the GenBank database using BLASTn.

For the Y2H assay, the DNA fragments of *triP*, *triP-N*, *triP-C*, *pdeR-G*, *pdeR-E*, and *pdeR-R* were amplified using primer pairs ADTriP-F/R, ADTriP-NF/R, BKTriP-NF/R, ADTriP-CF/R, BKPdeR-GF/R, ADPdeR-EF/R, and BKPdeR-RF/R, respectively. PCR products were cloned into pMD18-T for sequencing. The fragments were then digested from pMD18-T-derived constructs and were subcloned into prey vector pGADT7 or bait vector pGBKT7, resulting in pGADTriP, pGADTriP-N, pGADTriP-C, pGADPdeR-E, pGBKPdeR-R, pGBKPdeR-G, pGBKPdeR-E, and pGBKTriP-N (Table 1). The constructs were transformed into the *Saccharomyces cerevisiae* Y187 and Y2HGold strains, respectively, and were confirmed by PCR. Pairwise yeast mating between haploid cells, each containing pGADT7 and pGBKT7 constructs, was performed in yeast potato dextrose agar at 28°C for 24 h, then, the diploid cells were selected on DDO plates (SD/-Leu/-Trp) and were further screened on QDO/X/A plates (SD/-Leu/-Trp/-Ade/-His/X-α-Gal/AbA). Diploids were suspended in sterile water and four 10-fold serial dilutions were placed on the QDO plates. Growth of the diploids on QDO plates for 2 days indicated strong interactions between the test proteins.

Protein expression and purification.

The full length of *triP* was amplified by primer pair GEXTriP-F/R, was ligated into expression vector pGEX-6P-1, and was transformed into the *E. coli* BL21 strain for GST-tagged protein expression. In addition, full-length *pdeR* and *pdeK* was cloned into expression vector pET28a for expression as His₆-tagged fusions (Yang et al. 2012).

Fig. 8. A proposal model outlining TriP regulation of virulence expression via interacting with PdeR in *Xanthomonas oryzae* pv. *oryzae*. The response regulator PdeR has been shown to function as a phosphodiesterase (PDE) for cyclic dimeric guanosine monophosphate (c-di-GMP) hydrolysis and form a two-component system with the histidine kinase PdeK (Yang et al. 2012). TriP, the putative response regulator with the REC and helix-turn-helix domains specifically interact with the PdeR but not PdeK. The REC domain of TriP and the EAL domain of PdeR are essential for the protein-protein interaction. Such an interaction significantly impacts PdeR bipolar localization and PDE ability to degrade c-di-GMP into pGpG, thereby regulating bacterial virulence and exopolysaccharides (EPS) production in a c-di-GMP signaling-dependent manner.

For purification of the GST-fusion proteins, the following protocol was used. In short, expression of the target protein was induced by the addition of isopropyl- β -D-thiogalactoside at a final concentration of 0.1 mM in the bacterial culture M9. The cells were chilled to 4°C and were collected by centrifugation. The cell pellets were resuspended in 10 mM Tris-HCl (pH 8.0), followed by sonication. The soluble protein fractions were collected by centrifugation and were mixed with pre-equilibrated glutathione sepharose 4B (GE Healthcare, Piscataway, NJ, U.S.A.). The proteins were washed with 1× phosphate buffered saline (PBS) (pH 7.3) and were eluted with an elution buffer

(pH 8.0). pET28a-His₆-fusion proteins were purified through a Ni-binding column. The soluble protein fractions were mixed with pre-equilibrated Ni₂-resin (GE Healthcare) and were eluted with a buffer containing 300 mM imidazole. The GST tag expressed from pGEX-6P-1 was purified using the same procedure as a negative control. Protein concentration was assayed using the Bradford method (CWBIO, Beijing).

GST pull-down assay.

Pull-down experiments were performed with purified recombinant proteins. In short, 0.1 mg of GST-tagged recombinant proteins

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	<i>supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan 1983
BL21	For protein expression	Tiangen Biotech Co.
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Wild-type strain, Philippine race 6, Cp ^r	Lab collection
Δ pdeR	<i>pdeR</i> gene-deletion mutant derived from PXO99 ^A , Gm ^r , Cp ^r	Yang et al. 2012
Δ triP	<i>triP</i> gene-deletion mutant derived from PXO99 ^A , Cp ^r	This study
Δ triP(pBTriP)	Δ triP containing plasmid pBTriP, Ap ^r , Cp ^r	This study
Δ triP Δ pdeR	<i>triP</i> and <i>pdeR</i> double-deletion mutant. Cp ^r	This study
Δ triP(pBPdeR-GFP)	Δ triP containing plasmid pBPdeR-GFP, Ap ^r , Cp ^r	This study
Δ pdeR(pBTriP)	Δ pdeR containing plasmid pBTriP, Ap ^r , Cp ^r	This study
<i>Saccharomyces cerevisiae</i>		
Y2HGold	<i>MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, Δgal4, Δgal80, LYS2::GAL1_{UAS}-Gall_{TATA}-His3, GAL2_{UAS}-Gal2_{TATA}-Ade2, URA3::MEL1_{UAS}-Mell_{TATA}, AUR1-C MEL1</i>	Clontech
Y187	<i>MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, Δgal4, Δgal80, met-, URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ, MEL1</i>	Clontech
Plasmids		
pMD18-T	Cloning vector, Ap ^r	TaKaRa, Tokyo
pET28a	Expression vector to generate a N-terminal His ₆ -tag, Km ^r	Novagen, Madison, WI, U.S.A.
pGEX-6P-1	Expression vector to generate a N-terminal GST-tag, Ap ^r	Lab collection
pKMS1	Suicidal vector carrying <i>sacB</i> gene for nonmarker mutagenesis, Km ^r	Li et al. 2011
pBBR1MCS-4	Broad-host range expression vector, Ap ^r	Kovach et al 1995
pGADT7	Yeast two-hybrid vector to create fusion protein containing the GAL4 activation domain, Ap ^r	Clontech
pGBKT7	Yeast two-hybrid vector to create fusion protein containing GAL4 DNA binding domain, Km ^r	Clontech
pGBKPdeR	pGBKT7 carrying full-length <i>pdeR</i> , Km ^r	Yang et al. 2012
pET28a	pET28a carrying full-length <i>pdeR</i> , Km ^r	Yang et al. 2012
pETPdeK	pET28a carrying full-length <i>pdeK</i> , Km ^r	Yang et al. 2012
pMDTriP	pMD18-T derivative carrying full-length <i>triP</i> , Ap ^r	This study
pMDPdeR	pMD18-T derivative carrying full-length <i>pdeR</i> , Ap ^r	This study
pMDTriP-N	pMD18-T derivative carrying the REC domain of <i>triP</i> , Ap ^r	This study
pMDTriP-C	pMD18-T derivative carrying the HTH domain of <i>triP</i> , Ap ^r	This study
pMDPdeR-G	pMD18-T derivative carrying the GGDEF domain of <i>pdeR</i> , Ap ^r	This study
pMDPdeR-E	pMD18-T derivative carrying the EAL domain of <i>pdeR</i> , Ap ^r	This study
pMDPdeR-R	pMD18-T derivative carrying the REC domain of <i>pdeR</i> , Ap ^r	This study
pMDGFP	pMD18-T derivative carrying full-length <i>GFP</i> , Ap ^r	This study
pMDPdeR-GFP	pMD18-T derivative carrying full-length <i>pdeR</i> (without stop codon), Ap ^r	This study
pMDTriP-GFP	pMD18-T derivative carrying full-length <i>triP</i> (without stop codon), Ap ^r	This study
pMDPdeK-GFP	pMD18-T derivative carrying full-length <i>pdeK</i> (without stop codon), Apr	This study
pGADTriP	pGADT7 carrying full-length <i>triP</i> , Ap ^r	This study
pGADTriP-C	pGADT7 carrying the DNA fragment of <i>triP</i> HTH domain, Ap ^r	This study
pGADTriP-N	pGADT7 carrying the DNA fragment of <i>triP</i> REC domain, Ap ^r	This study
pGADPdeR-E	pGADT7 carrying the DNA fragment of <i>pdeR</i> EAL domain, Ap ^r	This study
pGBKPdeR-E	pGBKT7 carrying the DNA fragment of <i>pdeR</i> EAL domain, Km ^r	This study
pGBKPdeR-G	pGBKT7 carrying the DNA fragment of <i>pdeR</i> GGDEF domain, Km ^r	This study
pGBKPdeR-R	pGBKT7 carrying the DNA fragment of <i>pdeR</i> REC domain, Km ^r	This study
pGBKTriP-N	pGBKT7 carrying the DNA fragment of <i>triP</i> REC domain, Km ^r	This study
pGEXTriP	pGEX-6P-1 carrying full-length <i>triP</i> , Ap ^r	This study
pKMTriP	pKMS1 derivative carrying a <i>triP</i> mutation, Km ^r	This study
pKMPdeR	pKMS1 derivative carrying a <i>pdeR</i> mutation, Km ^r	This study
pBTriP	pBBR1MCS-4 carrying the full-length of <i>triP</i> , Ap ^r	This study
pBGFP	GFP fragment ligated into vector pBBR1MCS-4, Ap ^r	This study
pBPdeR-GFP	pBGFP carrying full-length <i>pdeR</i> , Ap ^r	This study
pBTriP-GFP	pBGFP carrying full-length <i>triP</i> , Ap ^r	This study
pBPdeK-GFP	pBGFP carrying full-length <i>pdeK</i> , Ap ^r	This study

^a Ap^r, Cp^r, Gm^r, and Km^r indicate resistance to antibiotics ampicillin, cephalixin, gentamycin, and kanamycin, respectively. HTH = helix-turn-helix.

were mixed with 0.1 mg of His₆-tagged proteins and were adjusted to a 1-ml volume using PBS. After being thoroughly mixed at room temperature, the glutathione sepharose 4B beads (200 µl slurry) were added and incubated at 4°C overnight. Coeluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were analyzed by immunoblotting using anti-GST and anti-His monoclonal antibodies (Beijing Protein Innovation, Beijing). Primary antibodies were recognized by goat antimouse secondary antibodies conjugated with horseradish peroxidase, which were directly visualized by applying the TMB substrate (Promega, Beijing). The purified GST protein was incubated with the His₆-tagged protein and was subjected to the same procedures to serve as a negative control.

PDE colorimetric assay.

The PDE activity was tested as previously described (Bobrov et al. 2005; Yang et al. 2012). In brief, 5 mM bis (p-nitrophenyl) phosphate substrate and 20 µg of purified proteins were incubated at 37°C for 1.5 h in reaction buffer (50 mM Tris-HCl, 1 mM MnCl₂, pH 8.5). The hydrolysis of substrate was detected at 410 nm by spectrophotometer. To test the effect of TriP on PDE activity of PdeR, 200 µM ATP and 20 µg of PdeK were incubated at 25°C for 3 min in phosphorylation buffer (25 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, pH 8.0) to a volume of 100 µl. Then, 20 µg of purified PdeR protein and 20 µg of purified TriP protein were added and incubated at 25°C for 20 min. Finally, 5 mM bis (p-nitrophenyl) phosphate substrate were added for the PDE colorimetric reaction and were incubated at 37°C for 2 h; the final volume was 200 µl. The hydrolysis of substrate was detected at 410 nm by spectrophotometer. Bovine serum albumin was used as a negative control. The experiment was repeated three times with three technical replicates.

Gene deletion mutagenesis and complementation.

The mutant was generated using homologous recombination by the suicide vector pKMS1 (Li et al. 2011). The DNA fragments corresponding to downstream and upstream regions of *triP* and *pdeR* were amplified, using primer pairs KMTriPLF/R, KMTriPRF/R, KMPdeRLF/R, and KMPdeRRF/R, respectively, and were cloned to the middle vector pMD18-T for sequencing. The pMD18-T-derived constructs were digested with corresponding restriction enzymes and were ligated into the pKMS1 suicide vector, resulting in plasmids pKMTriP and pKMPdeR. For the $\Delta triP$ deletion mutant, the plasmid pKMTriP was introduced into PXO99^A by electroporation, while for the $\Delta triP \Delta pdeR$ double mutant, the plasmid pKMPdeR was introduced into the *triP* mutant. Potential mutants were selected on NAN medium (1% tryptone, 0.1% yeast extract, 0.3% peptone, 1.5% agar). The single colonies were inoculated into NBN broth (1% tryptone, 0.1% yeast extract, 0.3% peptone) for selection against the *sacB* gene marker. Bacterial cells were diluted to appropriate concentrations and were plated on NAS (1% tryptone, 0.1% yeast extract, 10% sucrose, 0.3% peptone, 1.5% agar) for selection of mutants. The mutant candidates that can grow on NAS but are sensitive to kanamycin were further confirmed by PCR. For complementation analysis, the coding region for full-length *triP* was amplified by primer pair BTriPF/R, ligated into vector pBBR1MCS-4 and was electroporated into the $\Delta triP$ mutant for complementation studies.

Determination of the intracellular c-di-GMP concentration.

Intracellular c-di-GMP concentrations were determined by LC-MS/MS, using a method that has been previously described

(Yuan et al. 2015). In brief, bacterial cells were cultured at 28°C in M210 overnight and were then subcultured into 100 ml of fresh media (1:100 dilution). The cultures were grown to mid-to-late log phase (OD₆₀₀ = 2.5). Cells were collected by centrifugation at 9,000 × g for 20 min and were then resuspend in 1 ml of cold extraction buffer by pipetting (40% methanol and 40% acetonitrile in 0.1 N formic acid). The mixture was incubated at -20°C overnight, before ultrasonication treatment. The supernatant was collected after centrifugation at max speed for 10 min twice. Supernatant of each sample (100 µl) was analyzed by LC-MS/MS. The experiment was independently repeated three times.

Subcellular protein localization.

The full length of the *gfp* gene was amplified by PCR using primer pair BGFPF/R and was ligated into a broad host-range vector pBBR1MCS-4, resulting in pBGFP. We used the primer pairs BpdeR-GFPF/R, BpdeK-GFPF/R, and BTriP-GFPF/R to clone *pdeR*, *pdeK*, and *triP*. The fragments were then subcloned into pBGFP, resulting in pBPdeR-GFP, pBPdeK-GFP, and pBTriP-GFP. The constructs were transformed into different PXO99^A strains. Expressions of fusion proteins were confirmed by Western blotting, using anti-GFP primary antibodies (Abmart, Shanghai, China). Goat antimouse immunoglobulin G horseradish peroxidase conjugate secondary antibody (Abmart) was applied to recognize the primary antibodies.

Samples were prepared for microscopy as previously described (Xue et al. 2018). Different strains were grown in M210 to an OD₆₀₀ of 0.8. Bacterial cells were diluted, 3 µl of cell suspension was spotted on a glass slide that was freshly coated with 1.0% agarose, and the suspension was covered with a coverslip. Fluorescence images were acquired using an Olympus BX61 microscope with GFP and fluorescein isothiocyanate filters and were recorded with a cooled CCD camera DP72 (Olympus, Tokyo) at subsaturating emission intensities. The subcellular localization of cells was determined as described previously (Shikuma et al. 2012). At least 10 fields of view, each containing more than 100 cells were observed. The experiment was independently repeated three times.

Virulence assays.

The susceptible rice variety *Oryza sativa* L. cv. Nipponbare was used to test the virulence of *X. oryzae* pv. *oryzae* strains. Virulence assays were performed on 6-week-old rice plants using the leaf-clip inoculation method (Ray et al. 2000). Different PXO99^A strains were incubated at 28°C with agitation (approximately 200 rpm) until they reached an OD₆₀₀ = 0.8 to 1.0 in the M210 medium with relevant antibiotics. The cells were collected by centrifugation and were resuspended in sterilized distilled water. The concentration of this suspension was adjusted to an OD₆₀₀ = 0.8. Symptoms were photographed and lesion lengths were measured for individual inoculated leaves 14 days after inoculation. Twenty leaves were inoculated for each strain. The experiment was independently repeated four times.

Bacterial growth curve assays.

The bacterial growth assay was performed as previously described (Tian et al. 2015). In brief, *X. oryzae* pv. *oryzae* wild type and mutant strains were grown in M210 liquid medium overnight at 28°C; then, these strains were diluted in M210 medium to a final cell density (OD₆₀₀ = 0.01). The diluted cells were cultured at 28°C with 200 rpm, and the bacterial population was measured after every 12 h. The experiment was independently repeated twice.

EPS production assays.

EPS production was assayed as previously described (Xue et al. 2018). The bacterial culture (OD₆₀₀ = 2.5) were centrifuged

at 8,000 rpm for 20 min. Then, the supernatant was harvested, two volumes of absolute ethanol were added, and the mixture was kept at -20°C overnight. The precipitated EPS was collected by centrifugation and was dried before determination of dry weight. For the plate assays, bacterial strains were grown in M210 until they reached an $\text{OD}_{600} = 0.8$, and 2 μl of bacterial culture was spotted onto a PSA plate without any antibiotic and was incubated at 28°C for 4 days before photos were taken for the colonies. The experiment was repeated three times.

Statistical analysis.

All analysis was performed using SPSS 14.0 (SPSS Inc., Chicago) and Fisher's least significant difference test was used to determine significant differences in the assays for PDE activity, disease lesion length, in vivo growth, and EPS production.

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LITERATURE CITED

- Abel, S., Chien, P., Wassmann, P., Schirmer, T., Kaever, V., Laub, M. T., Baker, T. A., and Jenal, U. 2011. Regulatory cohesion of cell cycle and cell differentiation through interlinked phosphorylation and second messenger networks. *Mol. Cell* 43:550-560.
- Bobrov, A. G., Kirillina, O., and Perry, R. D. 2005. The phosphodiesterase activity of the HmsP EAL domain is required for negative regulation of biofilm formation in *Yersinia pestis*. *FEMS Microbiol. Lett.* 247: 123-130.
- Bourret, R. B. 2010. Receiver domain structure and function in response regulator proteins. *Curr. Opin. Microbiol.* 13:142-149.
- Dahlstrom, K. M., Giglio, K. M., Collins, A. J., Sondermann, H., and O'Toole, G. A. 2015. Contribution of physical interactions to signaling specificity between a diguanylate cyclase and its effector. *MBio* 6: e01978-15.
- Fang, X., and Gomelsky, M. 2010. A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Mol. Microbiol.* 76: 1295-1305.
- Gao, R., and Stock, A. M. 2009. Biological insights from structures of two-component proteins. *Annu. Rev. Microbiol.* 63:133-154.
- Güvener, Z. T., and Harwood, C. S. 2007. Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Mol. Microbiol.* 66:1459-1473.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
- Hengge, R. 2016. Trigger phosphodiesterases as a novel class of c-di-GMP effector proteins. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371: 20150498.
- Hopkins, C. M., White, F. F., Choi, S. H., Guo, A., and Leach, J. E. 1992. Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* 5:451-459.
- Jones, C. J., Utada, A., Davis, K. R., Thongsomboon, W., Zamorano Sanchez, D., Banakar, V., Cegelski, L., Wong, G. C. L., and Yildiz, F. H. 2015. C-di-GMP regulates motile to sessile transition by modulating MshA pili biogenesis and near-surface motility behavior in *Vibrio cholerae*. *PLoS Pathog.* 11:e1005068.
- Jung, K., Fried, L., Behr, S., and Heermann, R. 2012. Histidine kinases and response regulators in networks. *Curr. Opin. Microbiol.* 15:118-124.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd, and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBRR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.
- Li, B., Tian, F., Chen, H., Wu, M., and He, C. 2013. [Binding of transcription regulator ClpXco to promoter of endoglucanase gene *engA_{oxo}* was inhibited by c-di-GMP in *Xanthomonas oryzae* pv. *oryzae*.] *Wei Sheng Wu Xue Bao* 53:1166-1171.
- Li, Y. R., Zou, H. S., Che, Y. Z., Cui, Y. P., Guo, W., Zou, L. F., Chatterjee, S., Biddle, E. M., Yang, C. H., and Chen, G. Y. 2011. A novel regulatory role of HrpD6 in regulating *hrp-hrc-hpa* genes in *Xanthomonas oryzae* pv. *oryzicola*. *Mol. Plant-Microbe Interact.* 24:1086-1101.
- Lindenberg, S., Klauck, G., Pesavento, C., Klauck, E., and Hengge, R. 2013. The EAL domain protein YciR acts as a trigger enzyme in a c-di-GMP signalling cascade in *E. coli* biofilm control. *EMBO J.* 32: 2001-2014.
- Merritt, J. H., Ha, D. G., Cowles, K. N., Lu, W., Morales, D. K., Rabinowitz, J., Gitai, Z., and O'Toole, G. A. 2010. Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *MBio* 1:e00183-10.
- Niño-Liu, D. O., Ronald, P. C., and Bogdanove, A. J. 2006. *Xanthomonas oryzae* pathogens: Model pathogens of a model crop. *Mol. Plant Pathol.* 7:303-324.
- Paul, K., Nieto, V., Carlquist, W. C., Blair, D. F., and Harshey, R. M. 2010. The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. *Mol. Cell* 38:128-139.
- Paul, R., Jaeger, T., Abel, S., Wiederkehr, I., Folcher, M., Biondi, E. G., Laub, M. T., and Jenal, U. 2008. Allosteric regulation of histidine kinases by their cognate response regulator determines cell fate. *Cell* 133:452-461.
- Povolotsky, T. L., and Hengge, R. 2012. 'Life-style' control networks in *Escherichia coli*: Signaling by the second messenger c-di-GMP. *J. Biotechnol.* 160:10-16.
- Ray, S. K., Rajeshwari, R., and Sonti, R. V. 2000. Mutants of *Xanthomonas oryzae* pv. *oryzae* deficient in general secretory pathway are virulence deficient and unable to secrete xylanase. *Mol. Plant-Microbe Interact.* 13:394-401.
- Roelofs, K. G., Jones, C. J., Helman, S. R., Shang, X., Orr, M. W., Goodson, J. R., Galperin, M. Y., Yildiz, F. H., and Lee, V. T. 2015. Systematic Identification of cyclic-di-GMP binding proteins in *Vibrio cholerae* reveals a novel class of cyclic-di-GMP-binding ATPases associated with type II secretion systems. *PLoS Pathog.* 11:e1005232.
- Römling, U., Galperin, M. Y., and Gomelsky, M. 2013. Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77:1-52.
- Römling, U., Liang Z.-X., and Dow, J. M. 2017. Progress in understanding the molecular basis underlying functional diversification of cyclic dinucleotide turnover proteins. *J. Bacteriol.* 199:e00790-16.
- Schirmer, T., and Jenal, U. 2009. Structural and mechanistic determinants of c-di-GMP signalling. *Nat. Rev. Microbiol.* 7:724-735.
- Shikuma, N. J., Fong, J. C., and Yildiz, F. H. 2012. Cellular levels and binding of c-di-GMP control subcellular localization and activity of the *Vibrio cholerae* transcriptional regulator VpsT. *PLoS Pathog.* 8: e1002719.
- Simm, R., Morr, M., Kader, A., Nimtz, M., and Römling, U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* 53:1123-1134.
- Skerker, J. M., Prasol, M. S., Perchuk, B. S., Biondi, E. G., and Laub, M. T. 2005. Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: A system-level analysis. *PLoS Biol.* 3:e334.
- Sondermann, H., Shikuma, N. J., and Yildiz, F. H. 2012. You've come a long way: c-di-GMP signaling. *Curr. Opin. Microbiol.* 15:140-146.
- Stock, A. M., Robinson, V. L., and Goudreau, P. N. 2000. Two-component signal transduction. *Annu. Rev. Biochem.* 69:183-215.
- Sun, Q. H., Hu, J., Huang, G. X., Ge, C., Fang, R. X., and He, C. Z. 2005. Type-II secretion pathway structural gene *xpsE*, xylanase- and cellulase secretion and virulence in *Xanthomonas oryzae* pv. *oryzae*. *Plant Pathol.* 54:15-21.
- Tian, F., Yu, C., Li, H., Wu, X., Li, B., Chen, H., Wu, M., and He, C. 2015. Alternative sigma factor RpoN2 is required for flagellar motility and full virulence of *Xanthomonas oryzae* pv. *oryzae*. *Microbiol. Res.* 170:177-183.
- White, F. F., and Yang, B. 2009. Host and pathogen factors controlling the rice-*Xanthomonas oryzae* interaction. *Plant Physiol.* 150:1677-1686.
- Xue, D. R., Tian, F., Yang, F. H., Chen, H. M., Yuan, X. C., Yang, C.-H., Chen, Y. H., Wang, Q., and He, C. Y. 2018. Phosphodiesterase EdpX1 promotes *Xanthomonas oryzae* pv. *oryzae* virulence, exopolysaccharide production, and biofilm formation. *Appl. Environ. Microbiol.* 84: e01717-e01718.
- Yang, F., Qian, S., Tian, F., Chen, H., Hutchins, W., Yang, C.-H., and He, C. 2016. The GGDEF-domain protein GdpX1 attenuates motility, exopolysaccharide production and virulence in *Xanthomonas oryzae* pv. *oryzae*. *J. Appl. Microbiol.* 120:1646-1657.
- Yang, F., Tian, F., Chen, H., Hutchins, W., Yang, C.-H., and He, C. 2015. The *Xanthomonas oryzae* pv. *oryzae* PilZ-domain proteins function differentially in cyclic di-GMP binding and regulation of virulence and motility. *Appl. Environ. Microbiol.* 81:4358-4367.
- Yang, F., Tian, F., Li, X., Fan, S., Chen, H., Wu, M., Yang, C.-H., and He, C. 2014. The degenerate EAL-GGDEF domain protein Filp functions as a

- cyclic di-GMP receptor and specifically interacts with the PilZ-domain protein PXO_02715 to regulate virulence in *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 27:578-589.
- Yang, F., Tian, F., Sun, L., Chen, H., Wu, M., Yang, C.-H., and He, C. 2012. A novel two-component system PdeK/PdeR regulates c-di-GMP turnover and virulence of *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 25:1361-1369.
- Yuan, X., Khokhani, D., Wu, X., Yang, F., Biener, G., Koestler, B. J., Raicu, V., He, C., Waters, C. M., Sundin, G. W., Tian, F., and Yang, C.-H. 2015. Cross-talk between a regulatory small RNA, cyclic-di-GMP signalling and flagellar regulator FlhDC for virulence and bacterial behaviours. Environ. Microbiol. 17:4745-4763.
- Zhang, H., and Wang, S. 2013. Rice versus *Xanthomonas oryzae* pv. *oryzae*: A unique pathosystem. Curr. Opin. Plant Biol. 16:188-195.
- Zhou, L., Huang, T. W., Wang, J. Y., Sun, S., Chen, G., Poplawsky, A., and He, Y. W. 2013. The rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* produces 3-hydroxybenzoic acid and 4-hydroxybenzoic acid via XanB2 for use in xanthomonadin, ubiquinone, and exopolysaccharide biosynthesis. Mol. Plant-Microbe Interact. 26: 1239-1248.
- Zschiedrich, C. P., Keidel, V., and Szurmant, H. 2016. Molecular mechanisms of two-component signal transduction. J. Mol. Biol. 428: 3752-3775.

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