INTRODUCTION

Pathogens can transit rapidly between planktonic motile and sessile nonmotile phenotypes to adapt to changing environmental conditions. Motility allows bacteria to detect and pursue nutrients, and to reach and remain in their preferred habitats for colonization (Fenchel, 2002; Kimkes & Heinemann, 2020; Soutourina & Bertin, 2003). Motility plays a key role in various bacterial biological functions such as conjugation, chemotaxis, biofilm formation, and virulence. Bacterial motility is usually associated with flagella, which are complex surface
structures serving as the primary way of movement in many bacterial pathogens, allowing them to adhere to and invade host cells (Duan et al., 2013; Josenhans & Suerbaum, 2002; Macnab, 2003; Nakamura & Minaminno, 2019; Ramos et al., 2004). In contrast, under unfavourable conditions, bacteria can aggregate to form biofilms and become sessile, which protects them from various stress conditions such as nutrient depletion, conventional antimicrobial agents, and host immune reactions (Costerton et al., 1999; Yaron & Römling, 2014; Yin et al., 2021). Biofilm formation is a common characteristic of sessile bacteria, in which bacterial cells are encased by an extracellular matrix of exopolysaccharides (EPSs), proteins, and extracellular DNA (eDNA; Campoccia et al., 2021; Donlan, 2002; O’Toole et al., 2000; Römling et al., 2014). It has now been established that biofilms are critical for the majority of bacterial chronic infections (Gupta et al., 2016; Jamal et al., 2018). Understanding the molecular mechanisms underlying the motile-to-sessile transition in pathogens is therefore essential for the development of novel strategies for clinical treatments. To switch between these two fundamental lifestyles, bacteria have evolved various regulatory mechanisms including transcriptional factors (Baraquet et al., 2012; Soutourina & Bertin, 2003), quorum-sensing regulators (Clarke & Sperandio, 2005; Singh et al., 2000), small RNA (Irie et al., 2010; Majdalani et al., 2005), and the second messenger cyclic di-GMP (c-di-GMP; Valentini & Filloux, 2019).

Previous studies reported that c-di-GMP, a universal second messenger widely conserved in the bacterial kingdom, is implicated in regulation of diverse biological processes, especially in modulation of bacterial motile and sessile lifestyle transitions (Jenal et al., 2017; Romling et al., 2013; Ross et al., 1987; Valentini & Filloux, 2019; Wolfe & Visick, 2008; Yoon & Waters, 2021). Turnover of c-di-GMP in bacterial cells is modulated by two types of enzymes with opposite functions: diguanylate cyclase (DGC) and c-di-GMP-specific phosphodiesterase (PDE). DGC contains a GGDEF motif for c-di-GMP synthesis, whereas PDE contains an EAL or HD-GYP motif for c-di-GMP degradation (Jenal et al., 2017; Valentini & Filloux, 2019; Yoon & Waters, 2021). Generally, high intracellular c-di-GMP concentration promotes extracellular matrix production and subsequent biofilm formation, whereas low intracellular c-di-GMP level increases flagella-mediated motility (Povolotsky & Hengge, 2012; Romling et al., 2013; Simm et al., 2004; Thompson & Malone, 2020). Signalling of c-di-GMP is delivered downstream by its binding to a wide range of receptors with regulatory functions at multiple levels, from the transcriptional to posttranscriptional level, from protein stability and function, to conformational change (Romling et al., 2013; Valentini & Filloux, 2016, 2019; Wolfe & Visick, 2008). Known receptors include the PilZ domain-containing proteins (Cheang et al., 2019; Merighi et al., 2007; Ryjenkov et al., 2006), transcriptional regulators such as FlrA and FleQ (Baraquet et al., 2012; Srivastava et al., 2013), and riboswitches that exert posttranscriptional control (Hengge, 2010; McKee et al., 2018; Sudarsan et al., 2008).

PilZ domain-containing proteins have been considered to be the most important c-di-GMP receptors (Amikam & Galperin, 2006; Cheang et al., 2019; Galperin & Chou, 2020; Ryjenkov et al., 2006). The name of the domain originated from the Pseudomonas aeruginosa PilZ protein, encoded by PA2960, a member of the type 4 pilus biosynthesis gene cluster (Alm et al., 1996). Structural analysis of PilZ revealed an RxxR motif and a [D(N)x]SxxG motif that are the main c-di-GMP binding sites (Ryjenkov et al., 2006). The PilZ domain is a versatile module that can regulate various functions in a c-di-GMP-dependent manner (Cheang et al., 2019; Galperin & Chou, 2020; Naaz et al., 2021; Romling et al., 2013). Most characterized PilZ domain-containing proteins are involved in modulating the switch between planktonic and sessile lifestyles (Cheang et al., 2019; Galperin & Chou, 2020). One of the well-studied PilZ domain proteins is the YcgR-like protein, known as the flagellar brake protein. In Escherichia coli, YcgR controls flagellar motility by interrupting the MotA–Flig interaction (Hou et al., 2020; Paul et al., 2010). Another conserved PilZ domain protein is BcsA, which is a cellulose synthase fused with a PilZ domain. Its catalytic function is activated on binding to c-di-GMP and consequentially produces high levels of cellulose to boost biofilm formation (Romley & Galperin, 2015; Zorraquino et al., 2013).

Dickeya oryzae (previously named Dickeya zeae) is the agent of rice bacterial foot rot disease, which causes severe economic losses in rice-growing regions (Wang et al., 2020). Previous studies revealed two groups of virulence determinants: phytotoxins, known as zeamines, and bacterial motility (Zhou et al., 2011). Bacterial motility is regulated by several mechanisms, including the acyl homoserine lactone (AHL) quorum-sensing system (Hussain et al., 2008), a polyamine-mediated host-pathogen communication system (Shi et al., 2019), and the second messenger c-di-GMP (Cheang et al., 2016). Specifically, deletion of all the genes encoding c-di-GMP-degrading enzymes leads to nonmotile, hyperbiofilm and avirulent phenotypes (Chen et al., 2020).

To understand how c-di-GMP regulates D. oryzae motility and virulence, we identified two genes encoding PilZ domain-containing proteins through genome analysis of D. oryzae strain EC1, which share about 35% and 40% similarity to the YcgR and BcsA of E. coli, respectively. Deletion analysis showed that YcgR in D. oryzae EC1 is mainly responsible for modulation of bacterial motility, whereas BcsA takes a dominant role in regulation of biofilm formation. Biochemical analysis confirmed that YcgR and BcsA could physically interact with c-di-GMP with strong affinity. Double deletion of ycgR and bcsA fully rescued the altered phenotypes of the PDE-free mutant 7APDTE that contains a high level of cellular c-di-GMP, indicating the synergistic roles of YcgR and BcsA in c-di-GMP signalling regulation of D. oryzae physiology and virulence. This study identifies two key components in the c-di-GMP signalling pathway and provides a useful framework for further characterization of the sophisticated regulatory mechanisms that govern the physiology and virulence of D. oryzae.

2 | RESULTS

2.1 Two genes encoding PilZ domain-containing proteins were found in the D. oryzae EC1 genome

The second messenger c-di-GMP can bind to several classes of effector proteins, and most of these proteins do not share sequence
or structural similarity with each other. Among them, PilZ domain-containing proteins were the first to be discovered and are well-studied c-di-GMP effectors with conserved binding motifs found in a range of bacterial species (Cheang et al., 2019; Galperin & Chou, 2020; Ryan et al., 2012; Ryjenkov et al., 2006). To characterize the signalling pathways of c-di-GMP in D. oryzae EC1, we searched for genes encoding PilZ domain-containing proteins through the Pfam program, which led to the identification of W909_08750 (NCBI accession no. AJC66149) and W909_19655 (NCBI accession no. AJC68175). The gene W909_08750 encodes a YcgR-like protein and W909_19655 encodes a BcsA-like protein. For the convenience of this discussion, these two proteins of strain EC1 were named YcgR and BcsA, respectively. YcgR shares about 35% amino acid identity with its counterpart (PDB: 5Y6F_A) in E. coli K-12 (Table 1), which is known to be a flagellar brake protein involved in motility inhibition (Hou et al., 2020; Paul et al., 2010). BcsA from strain EC1 exhibits over 40% protein identity with homologs in E. coli K-12 and Salmonella typhimurium (Table 1), which represent another family of well-studied PilZ domain-containing proteins involved in cellulose biosynthesis and translocation (Romling & Galperin, 2015).

Protein domains were predicted by the simplified modular architecture research tool (SMART) and illustrated by DOG 2.0 software (Ren et al., 2009). YcgR contains YcgR and PilZ domains located in the N-terminus and C-terminus, respectively (Figure 1a). BcsA comprises a transmembrane (TM) domain with five segments at the N-terminus, a glycosyltransferase/cellulose synthetase domain in the middle, and a PilZ domain at the C-terminus (Figure 1a). The conserved RxxxR and (D/N)xSxxG motifs, which are essential for c-di-GMP binding affinity and stoichiometry, were identified in YcgR and BcsA through multiple sequence alignment with 10 characterized PilZ domain-containing proteins from different bacterial species (Figure 1b and Table 1).

2.2 Deletion of ycgR partially rescued bacterial motility and biofilm formation of a PDE-free mutant

Our previous study showed that the PDE-free mutant 7ΔPDE of D. oryzae EC1, in which all the seven genes encoding PDE enzymes were deleted, with c-di-GMP being highly accumulated in cells, became nonmotile and formed hyperbiofilms (Chen et al., 2020). To understand how the accumulated c-di-GMP caused these phenotype changes, the ycgR gene was deleted in the mutant 7ΔPDE and wild-type strain EC1. Motility assay results showed that deletion of ycgR in the nonmotile mutant 7ΔPDE restored its swimming and swarming motility to approximately 54% and 58% of the wild-type EC1 level, respectively. On the other hand, the swimming and swarming ability of single deletion mutant ΔycgR were not affected compared with its parent strain EC1 (Figure 2a,b). Complementation of 7ΔPDE with a heterologous PDE, RocR from P. aeruginosa PA01 (Rao et al., 2008), and the mutant 7ΔPDEΔycgR with ycgR both restored bacterial motility, identical to the phenotype of their parental strain wild-type EC1 and 7ΔPDE (Figure 2a,b). In contrast, in trans expression of the wild-type ycgR in the mutant ΔycgR abolished its motility similar to that of a flhCD mutant (Figure 2a,b). Given that the 7ΔPDE mutant forms hyperbiofilms, the effect of YcgR on the sessile phenotype of 7ΔPDE was also determined. Notably, deletion of the ycgR gene from 7ΔPDE decreased its biofilm mass by over 60% and increased cellulose production by about 40% (Figure 2c,d). Single knockout of ycgR in EC1 slightly decreased the biofilm formation and increased cellulose production by about 2.6-fold (Figure 2c,d). Similarly, expression of the heterologous PDE and wild-type ycgR in 7ΔPDE and 7ΔPDEΔycgR, respectively, could alter the biofilm and cellulose production (Figure 2c,d), whereas in trans expression of ycgR in the mutant ΔycgR increased these sessile phenotypes (Figure 2c,d). Considering that cellulose production was significantly increased after knockout of ycgR in either 7ΔPDE or EC1 backgrounds, transcriptional levels of cellulose biosynthesis genes were explored in ΔycgR and 7ΔPDEΔycgR strains, which showed no significant change compared to the levels in their parental strains wild-type EC1 and 7ΔPDE, respectively (Figure S1).

2.3 Deletion of bcsA decreased biofilm production in the PDE-free mutant

The function of another PilZ domain-containing protein BcsA, which belongs to the cellulose synthetase catalytic subunit A family (Romling & Galperin, 2015), was then explored in EC1 and the PDE-free mutant 7ΔPDE. In contrast to the role of YcgR in modulation of bacterial motility, deletion of bcsA in the nonmotile mutant 7ΔPDE did not alter the swimming motility or swimming ability of 7ΔPDE (Figure 3a,b). However, the single deletion mutant ΔbcsA significantly increased swarming ability while swimming motility decreased no more than 20% compared with the wild-type EC1 (Figure 3a,b). Complementation of ΔbcsA with the wild-type bcsA restored the

| Characteristics | YcgR | BcsA |
|-----------------|------|------|
| Amino acid identity with homolog in Escherichia coli K-12 (PDB: 5Y6F_A) | 35% | 46% |
| Amino acid identity with homolog in Salmonella typhimurium (NCBI accession no. MBJ4292000.1) | 39% | 48% |
| Amino acid identity with homolog in Dickeya dadantii 3937 (NCBI accession no. ADM98288.1) | 90% | 93% |
| RxxxR residue | RxxxR | RxxxR |
| (D/N)xSxxG residue | DxSxxG | NxSxxG |
bacterial motility to the EC1 level (Figure 3a,b). Deletion of bcsA in the hyperbiofilm mutant 7ΔPDE led to about a 60% decrease in biofilm mass (Figure 3c). Similarly, the single deletion mutant ΔbcsA had decreased biofilm production by over 50% compared with its parent strain EC1 (Figure 3c). In contrast to the negative effect of YcgR on cellulose production (Figure 2d), deletion of bcsA in strain EC1 decreased cellulose production to about 31%, while deletion of bcsA in the mutant 7ΔPDE slightly decreased the cellulose level (Figure 3d). Similarly, biofilm and cellulose production of the complementary strain ΔbcsA (BcsA) returned to wild-type levels, and in trans expression of bcsA in 7ΔPDE ΔbcsA increased the biofilm level although it was not completely recovered (Figure 2c,d). The above data showed that under the hyper-c-di-GMP conditions in mutant 7ΔPDE, deletion of bcsA did not cause a substantial impact on bacterial motility but did significantly rescue the hyperbiofilm phenotype. In contrast, under the same conditions, deletion of ycgR resulted in markedly enhanced motility and much reduced biofilm formation. Under the normal c-di-GMP conditions in the wild-type strain EC1, deletion of ycgR caused a greater impact than bcsA on cellulose production and less influence on biofilm formation and motility. Taken together, these data suggest that c-di-GMP might rely on these two effectors to modulate motile–sessile phenotypes.

2.4 YcgR and BcsA synergistically modulated motility and sessile phenotypes

To test whether these two PilZ domain-containing proteins could collaboratively regulate the bacterial phenotypes, the coding regions of ycgR and bcsA were deleted together in the mutant 7ΔPDE.
and the wild-type strain EC1. The results showed that double deletion of ycgR and bcsA in 7ΔPDE rescued its altered phenotypes of swimming, swarming, biofilm formation, and cellulose production, and in most cases restored these phenotypes to the wild-type levels (Figure 4). In complementation experiments, YcgR and BcsA were introduced into the 7ΔPDEΔycgRΔbcsA strain. Swimming, swarming motility, and cellulose mass of either 7ΔPDEΔycgRΔbcsA(YcgR) or 7ΔPDEΔycgRΔbcsA(BcsA) were reduced compared with the parental strain 7ΔPDEΔycgRΔbcsA, while biofilm formation was increased (Figure 4). On the other hand, double deletion of ycgR and bcsA in a wild-type EC1 background increased swimming motility, swarming motility, and cellulose production by about 37%, 40%, and 60%, respectively, compared with the wild-type level (Figure 4). Notably, compared with the wild-type EC1, biofilm formation of the ΔycgRΔbcsA mutant was decreased (Figure 4). As above, we preformed complementation experiments in the ΔycgRΔbcsA mutant by expressing in trans either ycgR or bcsA. Both swimming and swarming motility of ΔycgRΔbcsA(YcgR) increased significantly while ΔycgRΔbcsA(BcsA) generated a nonmotile phenotype, compared with the level of ΔycgRΔbcsA (Figure 4a,b). In the sessile phenotypes, compared with ΔycgRΔbcsA, biofilm and cellulose values of either ΔycgRΔbcsA(YcgR) or ΔycgRΔbcsA(BcsA) were reduced (Figure 4c,d). In our previous study, 7ΔPDE exhibited a nonmotile phenotype due to the reduced transcript levels of flagellum biosynthesis genes and the inability to assemble flagella (Chen et al., 2020). In the current study, single deletion of either ycgR or bcsA and their double deletion in 7ΔPDE mutant increased flagellar numbers as well as the transcriptional level of the master regulator flhCD (Figure S2). In addition, the gene expression level of bcsA measured in ΔycgR and 7ΔPDEΔycgR showed no differences compared with the value of their parental strains, and similar results were observed for ycgR in ΔbcsA and 7ΔPDEΔbcsA (Figure S3).
Inactivation of BcsA and YcgR restored the virulence of mutant 7ΔPDE

Our previous study showed that hyper-c-di-GMP mutant 7ΔPDE lost the ability to move and was unable to inhibit rice seed germination, suggesting that bacterial motility is a key virulence determinant (Chen et al., 2020). Given that in-frame deletion of ycgR and bcsA in mutant 7ΔPDE restored motile and sessile phenotypes to the wild-type level, we tested whether the recovery of these phenotypes was related to the virulence of mutant 7ΔPDE. The mutant 7ΔPDE and its derived mutants were subjected to pathogenicity tests using the rice seed germination assay. The results showed that deletion of bcsA in mutant 7ΔPDE slightly increased its virulence (seed germination inhibition rate) at two inoculant densities (10^4 and 10^5 cfu/ml; Figure 5a and Table S3). Deletion of ycgR in the same mutant resulted in a much more significant increase in its virulence than deletion of bcsA (Figure 5a and Table S3). Significantly, double deletion of bcsA and ycgR in the 7ΔPDE background fully restored its virulence against rice seeds, comparable to that of wild-type EC1 (Figure 5a and Table S3).

To visualize bacterial infection and colonization in rice seeds, a plasmid expressing the gfp (green fluorescent protein) gene was introduced to wild-type strain EC1 and its derivatives as described previously (Chen et al., 2020). The results showed that after inoculation with 10^6 cfu/ml bacterial cells for 40 h, the mutant 7ΔPDE and 7ΔPDEΔbcsA cells were hardly visible inside rice seeds, while the fluorescent cells of mutant 7ΔPDEΔycgR, and in particular wild-type and mutant 7ΔPDEΔycgRΔbcsA, were readily visible (Figure 5b). Consistent with the results of the fluorescence assay, the numbers of invading bacterial cells within rice seeds inoculated with mutants 7ΔPDE and 7ΔPDEΔbcsA were at similar very low levels, whereas the numbers of cells of mutants 7ΔPDEΔycgR and 7ΔPDEΔycgRΔbcsA reached about 50% and 80% of those inoculated with wild-type EC1, respectively (Figure 5c).

Notably, based on the wild-type EC1 background, single mutation of ycgR did not affect seed invasion and germination inhibitory
ability, whereas mutation of bcsA reduced seed invasive ability but not germination inhibitory ability (Figure S4). Analysis showed no significant difference in zeamine production in all the deletion mutants compared with wild-type EC1 (Figure S5). Moreover, the intracellular c-di-GMP level of 7ΔPDEΔycgR, 7ΔPDEΔbcsA, and 7ΔPDEΔycgRΔbcsA showed levels comparable to that of 7ΔPDE (Figure S6). These findings indicate that lack of YcgR or/but and BcsA proteins in 7ΔPDE restored its virulence ability without affecting zeamine production and c-di-GMP levels.

2.6 | YcgR and BcsA interaction with c-di-GMP is essential for their functionality

The binding ability of YcgR and BcsA with c-di-GMP was determined by isothermal titration calorimetry (ITC). Purified YcgR-GST was titrated with c-di-GMP at 25°C at a 1:1 stoichiometric ratio. The dissociation constant ($K_d$) was determined after analysis of the normalized ITC curve using MicroCal Origin software (http://originlab.com), which gave a value of $0.69 \pm 0.57 \mu M$ (Figure 6a). No binding was observed between c-di-GMP and the glutathione S-transferase (GST) tag (Figure S7). Previous studies reported that the first arginine in the RxxxR motif is directly involved in c-di-GMP binding (Ryjenkov et al., 2006; Yuan et al., 2015). To validate the role of this conserved motif of YcgR in c-di-GMP binding, the first arginine (R) of the RxxxR motif at position 124 was substituted by aspartic acid (D). This substitution abolished the binding ability of YcgR with c-di-GMP (Figure 6b).

To explore the interaction of the PilZ domain in BcsA protein with c-di-GMP, we expressed the PilZ domain of BcsA with a His-tag for ITC analysis. The ITC results showed that BcsA(PilZ) protein was capable of binding c-di-GMP at a 2:1 stoichiometric ratio with an estimated $K_d$ of $0.98 \pm 0.69 \mu M$ (Figure 6c). Consistent with the result.
for YcgR, after substituting the first arginine with aspartic acid in the RxxxR motif, the mutated derivative BcsA(PilZ)\textsubscript{R556D} was unable to bind c-di-GMP (Figure 6d).

The impact of the conserved c-di-GMP binding motif RxxxR on the biological activity of YcgR and BcsA was tested by analysis of swimming motility and the rice seed germination inhibition rate. The results showed that complementation of the mutant 7ΔPDE\textsubscript{ΔycgR} with wild-type ycgR abolished the bacterial motility and reduced the inhibitory effect on rice seed germination, identical with phenotypes of mutant 7ΔPDE\textsubscript{ΔycgR} (Figure 7a,b). In contrast, in trans expression of the R124D-substituted variant YcgR\textsubscript{R124D} in the mutant 7ΔPDE\textsubscript{ΔycgR} failed to restore these phenotypes (Figure 7a,b). Similarly, only expression of the wild-type bcsA could alter the swimming motility and rice seed germination inhibition, whereas in trans expression of its site-substituted variant bcsA\textsubscript{R556D} failed to change these phenotypes of 7ΔPDE\textsubscript{ΔycgRΔbcsA} (Figure 7a,b).

3 | DISCUSSION

Our previous study showed that c-di-GMP plays a critical role in the modulation of \textit{D. oryzae} motile–sessile phenotypes and virulence with unknown signalling mechanisms. In the current study, two PilZ domain-containing proteins were identified as the key c-di-GMP receptors in \textit{D. oryzae} EC1, namely YcgR and BcsA. YcgR plays a major role in regulation of bacterial motility and biofilm formation, whereas BcsA predominantly controls bacterial biofilm formation. Significantly, consistent with our previous findings that motility is a key virulence determinant, the results from this study showed that null mutation of ycgR in the hyper-c-di-GMP mutant 7ΔPDE generated a much more significant impact than deletion of bcsA on the mutant virulence against rice seeds (Figure 5). YcgR and BcsA were found to be able to bind to c-di-GMP and the first arginine within the conserved RxxxR motif of the PilZ domain was required for
c-di-GMP binding and their functionality. In particular, double deletion of ycgR and bcsA largely rescued the altered phenotypes of the mutant 7ΔPDE, indicating a synergistic role of these two PilZ domain-containing proteins in the c-di-GMP signalling pathway. Taken together, the data from this study demonstrate that c-di-GMP relies on YcgR and BcsA as effectors to modulate the bacterial motile–sessile phenotypes and virulence (Figure 8).

The findings showed that deletion of ycgR in the high c-di-GMP strain 7ΔPDE partially restored its motility and biofilm formation compared with its parental strain (Figure 2), which is similar to the
findings from *Salmonella*, *E. coli* and *Dickeya dadantii* 3937 in which deletion of *ycgR* in corresponding hyper-c-di-GMP mutants abolished the c-di-GMP-mediated swimming inhibition (Fang & Gomelsky, 2010; Yuan et al., 2015; Zorraquino et al., 2013). Notably, knockout of *ycgR* in either the ΔPDE or wild-type background reduced biofilm mass, but increased cellulose formation (Figure 2). However, reverse transcription-quantitative PCR (RT-qPCR) data showed no significant differences in the expression levels of the bacterial cellulose biosynthesis (*bcs*) cluster genes associated with cellulose biosynthesis in mutants ΔycgR and ΔPDEΔycgR compared with the parental wild-type strain EC1 and ΔPDE, respectively (Figure S1). We inferred that after deletion of *ycgR* in these strains, the cellular c-di-GMP concentration is in excess, markedly enhancing the amount of BcsA–c-di-GMP complex, resulting in elevated cellulose production. Another possibility is that YcgR may influence cellulose production through modulating a posttranscriptional pathway. Therefore, further research should be undertaken to investigate the relationship between biofilm formation and cellulose production, and the detailed regulation mechanisms of YcgR and BcsA on cellulose biosynthesis.

Protein interaction between YcgR and flagellar rotor proteins, such as Flig, has been reported in several enteric bacterial species (Cheang et al., 2019; Fang & Gomelsky, 2010; Hou et al., 2020; Le Guyon et al., 2015). However, bacterial two-hybrid and pull-down assays showed no interaction between YcgR with Flig in strain EC1 (our unpublished data). Considering that the hyper-c-di-GMP mutant ΔPDE failed to assemble flagella, with significantly decreased
transcription of flagellar genes (Chen et al., 2020), the current study explored the effect of knocking down the ycgR gene in 7ΔPDE on these phenotypes. Transmission electron microscopy and RT-qPCR analysis showed significant increases in flagellar biogenesis and much enhanced expression of the flagellar master regulatory genes flhCD in the mutant 7ΔPDEΔycgR compared with its parental strain 7ΔPDE (Figure S2). Multiple-sequence alignment with YcgR-like proteins whose PilZ domain possesses DNA-binding functions showed that the PilZ domain of YcgR in EC1 carries several positively charged amino acids associated with DNA binding (Figure S8). At this stage, we cannot rule out the possibility that YcgR in strain EC1 may function as a transcriptional factor in regulation of flagellar gene expression, in a way similar to other PilZ domain-containing proteins (Schumacher & Zeng, 2016; Wang et al., 2016; Wilksch et al., 2011). Taken together, these findings indicate that the ycgR gene may regulate bacterial flagella-mediated motility in a different mode, which awaits further investigation.

Deletion of bcsA in 7ΔPDE did not affect the bacterial motility phenotypes (Figure 3), which is in line with those of previous findings in Salmonella and D. dadantii 3937 (Yuan et al., 2015; Zorroquino et al., 2013). Notably, deleted bcsA in either 7ΔPDE or the wild-type EC1 background reduced biofilm formation as well as the cellulose level compared with the parental strain (Figure 3), in line with earlier studies that observed that cellulose is an exopolysaccharide component of biofilm matrix (Jahn et al., 2011; McCrate et al., 2013; Romling & Galperin, 2015). However, the biofilm and cellulose levels of 7ΔPDEΔbcsA remained significantly higher than that of wild-type EC1. It is possible that after the deletion of bcsA in the hyper-c-di-GMP mutant 7ΔPDE, the remaining high level of c-di-GMP might compensate for the loss caused by bcsA mutation in biofilm formation and cellulose production by activating other genes or pathways associated with these phenotypes. For example, in some enterobacteria, namely E. coli, Salmonella enterica, and Klebsiella pneumoniae, in addition to BcsA, BcsE is also involved in c-di-GMP-dependent cellulose biosynthesis (Fang et al., 2014). Sequence alignment did not find any BcsE homologs, but there are other genes within the bcs cluster of EC1 that were predicted to be involved in cellulose biosynthesis (Figure S9). Investigations on the cellulose synthesis pathway and biofilm architecture in strain EC1 might shed some light in understanding the regulatory mechanisms of c-di-GMP and its effector BcsA.

In Salmonella, deletion of two PilZ domain-containing receptors (YcgR and BcsA) effectively restores flagella-mediated motility in the ΔyhjH mutant (high c-di-GMP background strain), implying that cellulose is a suppressor of bacterial motility (Le Guyon et al., 2015; Zorroquino et al., 2013). However, different findings were observed in E. coli, where c-di-GMP-induced cellulose synthesis was not correlated with the YcgR-independent motility defect, but the regulator protein RssB was (Nieto et al., 2019). The findings from this study showed that mutant 7ΔPDEΔycgRΔbcsA effectively recovered the motile ability to the wild-type level (Figure 4), indicating that the function of BcsA in D. oryzae EC1 is similar to that in S. typhimurium, where cellulose may act as a flagellar “wheel lock” to inhibit swimming motility (Waters, 2013). In addition, this synergistic effect was observed for other phenotypes, including swarming motility, biofilm formation, and virulence, where double deletion of ycgR and bcsA in the 7ΔPDE mutant reversed these phenotypes to a much greater extent than the effect seen when ycgR and bcsA alone are deleted (Figure S10). Notably, the synergistic effect was not detected in the wild-type EC1 background, in which ycgR and bcsA did not seem to work together to affect all the phenotypes we tested (Figure S10). A possible explanation for these findings might be that elevated c-di-GMP levels drive YcgR and BcsA to work synergistically to control motile and sessile phenotypes, whereas in the wild-type background with normal c-di-GMP levels, YcgR and BcsA are responsible for respective functions. As YcgR-like and BcsA-like proteins are key for the control of virulence of EC1 under high c-di-GMP concentration, the findings in this study also provide new targets for the design of inhibitors for the subsequent control of soft rot diseases caused by Dickeya.

In summary, the findings from this study demonstrate that c-di-GMP in D. oryzae EC1 modulates motile–sessile phenotypes and virulence through two PilZ domain-containing proteins, YcgR and BcsA, that act as c-di-GMP receptors to transduce the c-di-GMP signal to regulate the expression of downstream genes. In D. dadantii 3937, which is closely related to D. oryzae EC1, c-di-GMP signalling regulates the type III secretion system (T3SS) and pectinase production in addition to motility and biofilm formation (Yi et al., 2010). Elevated c-di-GMP signalling regulates the expression of T3SS genes and pectate lyase (pel) production through YcgR3937 and BcsA3937 (Yuan et al., 2015). The findings from our previous study showed that c-di-GMP in D. oryzae EC1 mainly regulates motility and biofilm formation but had no effect on bacterial extracellular enzymes and zamine production (Chen et al., 2020). In this study, we further showed that elevated c-di-GMP levels did not influence the expression of T3SS genes (Figure S11). Taken together, these findings highlight the conservatism and plasticity of the c-di-GMP signalling pathway in different bacterial species and add new insight to the sophisticated regulatory mechanisms that govern D. oryzae physiology and virulence.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and plasmids

The details of the bacterial strains and plasmids used in this study are presented in Table S1. All E. coli strains were grown in LB medium at 37°C, whereas D. oryzae EC1 and its derivatives were cultured in Luri Bertani (LB) medium at 28°C. Minimal medium (MM) agar plates were used for all conjugation experiments (Hussain et al., 2008). L5S medium was used for determination of zamine production following a previously described method (Chen et al., 2016). Antibiotics were added at the following final concentrations: 100 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Km), 50 μg/ml streptomycin (Str), and 30 μg/ml polymyxin (Pm). The optical density (OD600) of bacterial cultures was determined using a NanoDrop 2000c (Thermo Fisher Scientific) at 600 nm.
4.2 | Construction of mutants and in trans complementation

Gene in-frame deletion and in trans complementation assays were performed using the suicide vector pKNG101 and broad-host expression plasmid pBRR1MCS-4, respectively, following a protocol described previously (Chen et al., 2016). The flanking region and the coding region of target genes were amplified through PCR using the specific primers listed in Table S2.

4.3 | Swimming and swarming motility assay

Collective swimming motility was assessed in a semisolid medium plate with 0.3% agar (Chen et al., 2016). An overnight bacterial culture was adjusted to OD<sub>600</sub> = 1.5 and a 1 µl sample was spotted onto the centre of the medium plate for 18-24 h at 28°C before determination of motility rate. Collective swarming motility rate was determined in a semisolid medium plate containing 0.4% agar (Chen et al., 2016). Bacteria were inoculated in the middle of the plate using a toothpick and incubated at 28°C for 16-24 h, then the diameter of the radial growth was measured.

4.4 | Biofilm formation assay

Biofilm formation assay was performed as described previously (Chen et al., 2020). Bacterial were incubated in glass test-tubes with SOBS (Super Optimal Broth with 1% sucrose) medium for 24 h and then stained with 0.1% (wt/vol) crystal violet. Biofilm mass was quantified using absorbance at 570 nm after decolourization with 70% ethanol.

4.5 | Assessment of cellulose levels

Cellulose production was quantified as described by Fang et al. (2014) with some modifications. A black 96-well microtitre plate with a glass bottom was inoculated with 200 µl of SOBS medium with 1.5% agar containing 50 µg/ml calcofluor white (Sigma-Aldrich). Eight microlitres of bacterial suspension (adjusted to the same OD<sub>600</sub>) was then added into each well and the plate was incubated at 28°C for 24-36 h under dark conditions. Fluorescence was determined using a Synergy H1 multilabel reader (excitation/emission wavelengths 355/460 nm) (BioTek).

4.6 | Protein expression and purification

The coding regions of ycgR and bcsA(PilZ) from EC1 were amplified with the primers listed in Table S2 and cloned into GST-tagged vector pGEX-6P-1 and the His-tagged vector pCold-SUMO, respectively. Recombinant plasmids were transformed into E. coli BL21 (DE3) for expression. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to obtain a final concentration of 0.5 mM. Cells were harvested and then resuspended in xTractor buffer (Clontech, Takara) for lysis. For GST-tag protein, affinity purification was performed at 4°C using Glutathione-Superflow Resin (Clontech, Takara), washed with phosphate-buffered saline (PBS), and the target protein was eluted using gradient elution buffer (50 mM Tris-HCl, pH 8.0) containing 33 mM reduced glutathione. For His-tag protein, affinity purification was performed at 4°C using TALON Metal Affinity Resin (Clontech, Takara), washed with equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole [pH 7.4]), then stained with 0.1% (wt/vol) crystal violet. Biofilm mass was quantified using absorbance at 570 nm after decolourization with 70% ethanol.

4.7 | Isothermal titration calorimetry

ITC analysis was performed using a MicroCal ITC200 calorimeter (GE Healthcare). Proteins and ligands were dissolved in the same binding buffer (300 mM NaCl, 0.5 mM EDTA, 10% glycerol, 50 mM sodium phosphate, pH 7.4). Ligands (250, 500, or 600 µM) were titrated into the sample cell containing target proteins (10 or 24 µM). Experimental parameters were as follows: 60 s of initial delay and 10 µcal/s reference power at 25°C under 1000 rpm constant stirring. One or two microlitres of c-di-GMP was titrated every 150 s, 40 times for BcsA(PilZ) and 20 times for BcsA(PilZ)<sub>R556D</sub>, YcgR, and YcgR<sub>R124D</sub>. The titration experiment was performed twice. ITC binding curves were fitted with the one-site model to determine the association constant (K<sub>a</sub>) using MicroCal ORIGIN v. 7.0 software (Origin Lab Corp.).

4.8 | Rice seed germination and bacterial invasion assays

The rice seed germination assay was performed following a method described previously with minor modifications (Chen et al., 2020). Bacterial cultures were diluted with PBS in a series of five cfu concentrations, from 10<sup>3</sup> to 10<sup>9</sup> cfu/ml. A total of 15 rice seeds (variety CO39) were immersed in 10 ml of each bacterial dilution culture for 6 h at 28°C. Seeds were then washed with sterile water and transferred to a Petri dish containing moistened filter papers at 28°C for a week. The experiment was performed in duplicate and the germination inhibition ratio was calculated.

Microscopy visualization assay was performed using bacterial cells containing a GFP expression plasmid as described previously (Chen et al., 2020). In brief, bacterial cells were harvested and resuspended in PBS, and adjusted to 10<sup>6</sup> cfu/ml. Rice seeds (variety CO39) were immersed in bacterial cultures for 6 h and incubated for 40 h at 28°C. The husks were removed and the seeds were observed...
using a DMi8 fluorescence inverted microscope under GFP and RFP filters (Leica), then used for invasion cfu calculation. Fluorescent photographs were merged using Leica LAS-X software. The experiment was performed in duplicate.

For invasive cfu calculation, the husks of at least five rice seeds were removed and ground to powder then resuspended with 1 ml sterile water. Samples were diluted appropriately and spread onto LB agar plates containing tetracycline. Samples were then cultured overnight for bacterial cfu counting. The experiments were performed in duplicate.

4.9 | Quantitative analysis of c-di-GMP by LC-MS

Extraction of c-di-GMP from bacterial cells was performed as described previously (Chen et al., 2020). Bacterial cells were lysed with perchloric acid (70% vol/vol) and then neutralized by addition of 2.5 M KHCO₃. Supernatants were stored at −80°C before c-di-GMP analysis by liquid chromatography-mass spectrometry (LC-MS).

c-di-GMP quantification was performed following a previous protocol with modifications (Chen et al., 2020). Five microliters of each sample was analysed using a Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) using a 1.8 μm, 100 × 2.1 mm high-strength silica (HSS) T3 column (ACQUITY UPLC, Waters). A gradient system was used for isocratic elution with 95% aqueous (2.5 mM ammonium acetate) and 5% organic (methanol), with a 0.2 ml/min flow rate and a 7 min cycle time. c-di-GMP was detected by Orbitrap mass analyser on a Q Exactive Focus system under positive ionization mode. The ion spray voltage was 4.2 kV, and the sheath gas and aux gas flow rates were set at 30 and 10 units, respectively. The m/z 691 > 248 transition was used for quantification; 691 > 152 and 691 > 540 were monitored as confirmatory signals, performed in parallel reaction monitoring (PRM) mode with resolution and AGC target at 35,000 and 5e4, respectively. The collision energy was 30 eV and the isolation window was 4.0 m/z.

For generation of a standard curve, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 nM of pure c-di-GMP (Biolog) with 0.1% formic acid were analysed as described above. c-di-GMP levels were normalized to total protein per millilitre of culture. Data were expressed as the means of three independent cultures with error bars indicating the standard deviation.

For protein quantification, precipitated fractions were resuspended in 1 M NaOH and heated in an ion bath. The protein lysate was quantified using a Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The experiment was performed in triplicate.

4.10 | Zeamine production assay

The zeamine production assay was carried out in L55 medium and E. coli DH5α was used as the indicator bacterium, as previously described (Chen et al., 2016). Zeamines (units) = 0.5484e0.886x (R² = 0.9957), where x is the width in millimetres of the growth inhibition zone surrounding each well.

4.11 | Examination of flagella using transmission electron microscopy

Bacterial specimens were stained with 2% (wt/vol) phosphotungstic acid and observed under a FEI Talos F200 microscope at 200 kV as described previously (Chen et al., 2020).

4.12 | RNA extraction and RT-qPCR assays

RNA extraction was conducted using TRIzol reagent and the Phasemaker Tubes Complete System (Thermo Fisher Scientific), as previously described (Chen et al., 2020). Thereafter, TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech) was used to synthesize the cDNA for each RNA sample. The qPCR assay was performed using Talent qPCR PreMix (SYBR Green) (Tiangen Biotech) and the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturers’ protocol. The internal reference gene was gapA and the relative level of gene expression was calculated using the ΔΔCₜ method, according to the MIQE guidelines (Taylor et al., 2010). The primers for the SYBR Green qPCR were designed using Beacon designer software (http://www.premierbiosoft.com/molecular_beacon/) and are listed in Table S2.

4.13 | Bacterial growth analysis

Overnight bacterial cultures grown in LB broth were diluted (1:100) in the same medium and adjusted to same cell density. Two hundred microlitres of the diluted culture were grown in each well at 28°C in a low-intensity shaking model using the Bioscreen-C Automated Growth Curves Analysis System (OY Growth Curves AB Ltd).

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CONFLICT OF INTEREST

Authors declare that there are no known conflicts of interest associated with this paper.
DATA AVAILABILITY STATEMENT
The genome sequence of D. oryzae EC1 is accessible in the NCBI database https://www.ncbi.nlm.nih.gov/ under accession no. NZ_CP006929.1.

ORCID
Yufan Chen https://orcid.org/0000-0001-9669-7380
Mingfa Lv https://orcid.org/0000-0003-4902-3716
Jianuan Zhou https://orcid.org/0000-0001-9774-330X

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