Integration Host Factor Is Essential for Biofilm Formation, Extracellular Enzyme, Zeamine Production, and Virulence in *Dickeya zeae*

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Dickeya zeae is a globally important pathogenic bacterium that infects many crops, including rice, maize, potato, and banana. Bacterial foot rot of rice caused by *D. zeae* is one of the most important bacterial diseases of rice in China and some Southeast Asian countries. To investigate the functions of integration host factor (IHF) in *D. zeae*, we generated knockout mutants of *ihfA* and *ihfB*. Phenotypic assays showed that both the $\Delta ihfA$ and $\Delta ihfB$ strains had greatly reduced mobility, biofilm formation, extracellular protease, and pectinase activities, and toxin production compared with the wild-type strain. In addition, the mutants did not inhibit the germination of rice seeds, failed to cause soft rot in potatoes and a hypersensitive response in tobacco, and were avirulent in rice. Quantitative reverse-transcription polymerase chain reaction analysis demonstrated that IHF positively regulates the expression of *zmsA*, *hrpN/Y*, *pelA/B/C*, *pehX*, *celZ*, *prtG*, *fliC*, and *DGC* (diguanylate cyclase). Electrophoretic mobility shift assays further confirmed that *IhfA* binds to the promoter region of the *DGC* gene and may alter the levels of a second bacterial messenger, c-di-GMP, to regulate the pathogenicity or other physiological functions of *D. zeae*. In summary, IHF is an important integrated regulator of pathogenicity in *D. zeae*.

Bacterial foot rot caused by *Dickeya zeae* (synonym *Erwinia chrysanthemi* pv. *zeae*) is one of the most serious diseases of rice. Since first being reported in Japan in the 1970s, outbreaks have occurred in the Philippines, India, Indonesia, Bangladesh, North Korea, and other southeast Asian countries, causing severe losses in rice production (Liu et al. 2013; Zhang et al. 2018). In addition to rice and maize, *D. zeae* also infects other economically important crops, such as potato and banana (Parkinson et al. 2009; Liu et al. 2015). Despite its importance, few studies have investigated the pathogenic mechanism of *D. zeae*. Quorum sensing is a widespread regulatory mechanism in plant-pathogenic bacteria and, in *D. zeae*, can modulate bacterial motility and the ability to form multicellular aggregates, ultimately affecting virulence (Hussain et al. 2008).

Recently, a novel polyamino-amide antibiotic, designated zeamine, with widespread antibacterial effect was identified in *D. zeae* (Wu et al. 2010). Further investigation indicated that zeamine, which is a phytotoxin, plays a key role in the pathogenicity of *D. zeae* in monocotyledonous plants (Cheng et al. 2013; Zhou et al. 2011). We have also previously shown that the two-component system HrpX/HrpY of *D. zeae* positively regulates motility, biofilm formation, and pathogenicity (Chen et al. 2014).

Integration host factor (IHF), which was first characterized as a λ -phage accessory protein involved in the site-specific recombination process (Miller and Friedman 1980), acts as a universal sequence-specific DNA-bending protein in many gram-negative bacteria (Dorman 2009; Muñoz et al. 2010). IHF is a small, heterodimeric protein of approximately 20 kDa and is encoded by the genes *ihfA* and *ihfB* (Haluzi et al. 1991). Generally, a 13-bp specific consensus target sequence (WATCAANNNTTTR, W means A or T, R means A or G, N means any nucleotide) is recognized and bound by IHF, which then carries out its biological function (Hirano 2006). IHF proteins from *Escherichia coli* and other animal-pathogenic bacteria are involved in the transcriptional regulation of over 100 genes with different functions (Arfin et al. 2000; Mangan et al. 2006). For example, in *Salmonella enterica* serovar typhimurium LT2, IHF provides essential regulation of the enzymes involved in propionate catabolism, which are encoded by the *prpBCDE* operon (Palacios and Escalante-Semerena 2000). IHF binds to the promoter sequence of the *virB* operon (the type IV secretion system), regulating the transcription of the downstream genes and thereby reducing the virulence of this pathogenic bacterium (Sieira et al. 2004). In *Shigella flexneri*, IHF positively regulates virulence gene expression through binding to multiple promoters, including those associated with *virF*, *virB*, and *iscA* (Porter and Dorman 1997). In addition, IHF plays a role in the regulation of virulence genes in *Vibrio cholerae*, including the inactivation of *ihfA* and *ihfB*, and reducing the expression of the two main virulence genes, *tcpA* and *ctx*, so as to hinder production of the cholera toxin (Stonehouse, et al. 2008).

The requirement of IHF for virulence has also been described in plant-pathogenic bacteria. For example, IHF binds to the *hrpLEcc* promoter in *Pectobacterium carotovora* subsp. *carotovora* (Chatterjee et al. 2002) and to the *hrpS* promoter in *Pantoea stewartii* subsp. *stewartii* (Merighi et al. 2006), thereby modulating virulence by positively regulating genes involved in the type III secretion systems of these species (Chatterjee et al. 2002; Merighi et al. 2006). In addition, the presence of IHF greatly reduces bean toxin production by plant pathogen *Pseudomonas syringae* pv. *phaseolicola* (Arvizu-Gómez et al.

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2011). Interestingly, while IHF is essential for RpoN-dependent *hrpL* expression in *Erwinia amylovora*, it can also control bacterial motility by positively regulating *rsmB* sRNA (Lee and Zhao 2016) and positively regulates *hrpS* expression through directly binding the *hrpX* promoter and positively regulating *hrpX/hrpY* expression (Lee and Zhao 2018). However, little is known about the role of IHF in *D. zeeae*.

In this study, *D. zeeae* $\Delta ihfA$ and $\Delta ihfB$ deletion mutant strains were generated and were subjected to phenotypic and gene expression analyses in comparison with the wild-type strain for understanding the role of IHF in the regulatory cascade controlling *D. zeeae* pathogenesis.

RESULTS

Mutations in *ihfA* and *ihfB* significantly affect the bacterial growth.

To characterize the function of IHF in *D. zeeae*, we successfully constructed single knockout mutant strains $\Delta ihfA$ and $\Delta ihfB$ along with their respective complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$ (Supplementary Fig. S1). To determine whether the IHF-encoding genes affect the growth and proliferation of *D. zeeae*, the growth of the wild-type, mutant, and complementation strains was compared in Luria Bertani (LB) medium. Compared with the wild type, both $\Delta ihfA$ and $\Delta ihfB$ initially grew much more slowly; however, both mutant strains reached cell densities similar to that of the wild type at 24 h post-inoculation. Complementation mutant strains R- $\Delta ihfA$ and R- $\Delta ihfB$ showed similar growth patterns to the wild type (Fig. 1).

IHF positively regulates motility and biofilm formation in *D. zeeae*.

Equal amounts of *D. zeeae* wild-type, mutant, and complementation strains were transferred onto semisolid agar plates and were cultured for 24 h. Mutant strains $\Delta ihfA$ and $\Delta ihfB$ exhibited decreased circular movement, with decreases in colony diameter

Fig. 1. Growth curves of *Dickeya zeeae* wild-type strain EC1, integration host factor (IHF) mutants, $\Delta ihfA$ and $\Delta ihfB$, and their complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$. The growth (optical density at 600 nm [OD₆₀₀]) of each strain, in Luria Bertani medium at 30°C, was measured at every 2 h. Datapoints represent the means of three replicates.

compared with the wild type of 37.5 and 71.9%, respectively. The complementation strains R- $\Delta ihfA$ and R- $\Delta ihfB$ produced similar results to the wild-type strain EC1 (Fig. 2).

Biofilms are bacterial communities attached to a surface. Life in these communities provides protection from deleterious conditions. Therefore, biofilm formation is considered important for the disease cycle of bacterial pathogens of both animals and plants. Both mutant strains showed a significant decrease in biofilm formation in glass tubes compared with the wild-type and complementation strains (Fig. 3).

IHF positively regulates the activities of extracellular enzymes and toxin in *D. zeeae*.

In many phyto bacteria, proteases, cellulases, and pectinases are pathogenic factors. The activities of these extracellular enzymes were, therefore, investigated in the *D. zeeae* wild-type,

Fig. 2. Motility of *Dickeya zeeae* wild-type strain EC1, integration host factor (IHF) mutants, $\Delta ihfA$ and $\Delta ihfB$, and their complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$. **A**, Two microliters of the bacterial suspensions (optical density at 600 nm [OD₆₀₀] = 1.5) were inoculated at the center of the motility plate with semisolid medium and were incubated for 24 h at 30°C to assess the movement of these strains on the motility plate. **B**, The diameters of the growth circle around the inoculation site were measured. Values are means of three replicates and the error bars are standard deviation. Statistical analysis was performed on all data together and significantly different values (analysis of variance $P < 0.05$) are indicated by different letters.

mutant, and complementation strains. The results showed that, compared with the wild type, the protease activity of $\Delta ihfA$ and $\Delta ihfB$ was decreased by 95 and 82%, respectively (Fig. 4A and E), while the pectinase activity of the two strains was also decreased by 62 and 65%, respectively. (Fig. 4B and E). However, no significant decreases in cellulase activity were observed for the two mutant strains compared with the wild-type (Fig. 4C and E), indicating that IHF positively regulates the activities of proteases and pectinases but not that of cellulases in *D. zeae*.

The toxins produced by wild-type *D. zeae* can inhibit the growth of *E. coli* on LB plates (Zhou et al. 2011). Therefore, tested strains were compared for bactericidal activity against *E. coli* on LB medium. Neither $\Delta ihfA$ nor $\Delta ihfB$ produced zones of inhibition on plates spread with *E. coli*, while the complemented strains R- $\Delta ihfA$ and R- $\Delta ihfB$ produced inhibition zones similar to those of the wild-type strain (Fig. 4D and E), suggesting that IHF is involved in toxin production in *D. zeae*.

IHF mutants lost the ability to inhibit rice seed germination.

Virulent *D. zeae* strains strongly inhibit the germination of rice seeds (Zhou et al. 2011). Comparison of the effects of the wild-type, mutant, and complementation mutant strains on rice seed germination showed that there was no significant difference between the mutant strains and the blank control in germination. In contrast, the complementation strains showed strong inhibition of rice seed germination that was comparable to that of the wild-type strain (Fig. 5). This indicated that deletion of the IHF-encoding genes reduced the ability of the bacterium to inhibit rice seed germination.

Deletion of IHF abolishes virulence in potato and rice and the hypersensitive response (HR) in tobacco.

To characterize the role of IHF in the virulence of *D. zeae*, the wild-type, mutant, and complementation mutant strains were inoculated onto potato, rice, and tobacco plants, which were then monitored for signs of disease. At 24 h postinoculation, the slices of potato inoculated with the wild-type, R- $\Delta ihfA$, or R- $\Delta ihfB$ strains showed signs of severe soft rot, while the potato slices inoculated with mutant strains $\Delta ihfA$ or $\Delta ihfB$ had very few symptoms of soft rot (Fig. 6A). This indicated that IHF is required for the development of *D. zeae*-induced soft rot in potatoes.

Symptoms of soft rot, including curling and wilt of interior leaves and watery brown spots on the leaf sheath at the base of the stem, were observed in rice seedlings inoculated with the wild-type or complementation strains at 48 h postinoculation. At 7 days postinoculation, the stem bases of rice seedlings inoculated with the wild-type or complementation mutant strains

turned black and rotten, giving off a putrid smell (Fig. 6B). However, no symptoms appeared in the rice seedlings inoculated with the mutants $\Delta ihfA$ and $\Delta ihfB$ (Fig. 6B). This implies that IHF is essential for *D. zeae* to cause foot rot on rice seedlings.

At 24 h postinoculation, no signs of a HR were present on tobacco leaves inoculated with mutant strains $\Delta ihfA$ or $\Delta ihfB$, while a HR was apparent on tobacco leaves inoculated with the wild-type, R- $\Delta ihfA$, or R- $\Delta ihfB$ strains (Fig. 6C). This result confirmed that IHF regulates the Hrp system in *D. zeae*.

IHF positively regulates the expression of some virulent genes in *D. zeae*.

To verify the downstream genes regulated by IHF, the expression of several target genes, including *zmsA* (encodes a novel multimodular polyketide synthase forzeamine toxin), *hrpN* and *hrpY* (Hrp system), pectate lyase genes (*pelA*, *pelB*, *pelC*), polygalacturonases (*pehX*), *celY* and *celZ* (cellulase), *prtG* (serralysin G), *fliC* (a regulatory gene in flagellum system), and *DGC* (diguanylate cyclase) in the *D. zeae* wild-type, $\Delta ihfA$, and $\Delta ihfB$ strains was compared, using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. The results showed that the expression of *zmsA*, *hrpN*, *hrpY*, *pelA*, *pelB*, *pelC*, *pehX*, *celZ*, *prtG*, *fliC*, and *DGC* was significantly decreased in the mutant strains compared with that in wild-type strain EC1 (Fig. 7). Interestingly, the decrease in gene expression was more noticeable in $\Delta ihfB$ than in $\Delta ihfA$. However, the expression of *celY* was significantly higher in $\Delta ihfA$. This indicates that IHF positively regulated the expression of several virulence genes in *D. zeae*.

IhfA binds to the DGC promoter.

IHF recognizes a specific asymmetric DNA sequence, WCARNWNNTTR, which is conserved in the IHF α -subunit-encoding gene *ihfA*. After binding to this sequence, IHF causes the DNA to bend at an angle of up to 180° at the 5' terminal region of the binding site (5'-WWWCAR-3') (Hirano 2006; Wagner 2000). To investigate this binding, IhfA was expressed and purified (Supplementary Fig. S2). Cyclic di-GMP is a second bacterial messenger that is synthesized from two molecules of GTP by the action DGC and is then hydrolyzed by specific c-di-GMP phosphodiesterases (PDE) (Schirmer and Jenal 2009). DGC can regulate virulence in some bacterial plant pathogens by modulating the intracellular c-di-GMP levels (Chatterjee et al. 2010; Edmunds et al. 2013; Su et al. 2016). Analysis of DGC promoter sequences identified a putative IHF-binding site (TTATCAGTTGATTA) in the promoter region (within the -214 bp box) upstream of the DGC open reading frame (Fig. 8A). To verify whether IhfA binds to this specific sequence in the DGC promoter, an electrophoretic mobility shift assay (EMSA) was

Fig. 3. Biofilm formation of *Dickeya zeae* wild-type strain EC1, $\Delta ihfA$, and $\Delta ihfB$, and their complementation strains R- $\Delta ihfA$ and R- $\Delta ihfB$. A 10- μ l aliquot of bacterial cell culture (optical density at 600 nm = 0.5) was inoculated into tubes containing SONG medium (per liter: 20 g of tryptone, 5 g of yeast extract, 5g of NaCl, 2.4 g of $MgSO_4 \cdot 7H_2O$, 0.186 g of KCl, 50 ml of 40% glycerin) and was incubated for 72 h at 28°C. $\Delta ihfA$ and $\Delta ihfB$ showed a significant decrease in biofilm formation in glass tubes compared with wild-type strain EC1 and the complemented strains.

performed using a probe targeting this region. The results demonstrated that IhfA could bind to the DGC promoter (Fig. 8B).

DISCUSSION

Virulence factors and secondary metabolites produced by plant-pathogenic bacteria are normally controlled via a strict process that

responds to multiple environmental cues. IHF is a global regulatory factor in some animal- and plant-pathogenic bacteria (Chatterjee et al. 2002; Lee and Zhao 2016; Mangan et al. 2006; Merighi et al. 2006; Seira et al. 2004; Stonehouse et al. 2008), in which it regulates many biological functions, including virulence (Arvizu-Gómez et al. 2011; Lee and Zhao 2016, 2018; Mangan et al. 2006; Stonehouse et al. 2008). However, little is known about the IHF

Fig. 4. Determination of extracellular enzymes and toxin of *Dickeya zeae* wild-type strain EC1, $\Delta ihfA$, and $\Delta ihfB$, and their complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$. **A**, Protease assay. 2 μ l of the bacterial suspensions were inoculated on the Luria Bertani plate with 2% defatted milk and were cultivated for 24 h at 30°C, to observe the hydrolysis zone around the colony. **B**, Pectinase assay. Bacterial suspensions (2 μ l) were inoculated on the pectinase test plate with 1% polygalacturonic acid and were cultivated for 24 h at 30°C, then, a 1-mol/liter HCl solution was poured into the plate and the transparent zone was observed. **C**, Cellulase assay. Bacterial supernatants (50 μ l) were added into the hole of a carboxymethyl ethyl cellulose plate and were incubated for 24 h at 30°C. The plate was then stained with 0.1% (wt/vol) Congo red for 30 min, was washed with 1 M NaCl, and the size of hydrolysis zone around the hole was observed. **D**, Toxin assay. Bacterial supernatants (50 μ l) were added into the hole of an *Escherichia coli* plate and were incubated for 24 h at 30°C, to observe the inhibition zone. **E**, A bar graph showing the measurement of diameters of hydrolysis zones observed in A and C or inhibition zone in D. The values are means of three replicates and the error bars are standard deviation. Statistical analysis was performed on each group of data and significantly different values (analysis of variance $P < 0.05$) are indicated by different letters.

proteins in the important plant pathogen *D. zea*. In this study, we demonstrated that IHF is essential for zeamine toxin production and virulence of *D. zea* and controls swimming motility, biofilm formation, and the expression of extracellular enzymes.

Bacterial motility is particularly important in the early stages of host invasion (Shen et al. 2001). In animal-pathogenic species *Yersinia enterocolitica* and *Shewanella oneidensis*, bacterial motility and biofilm formation are closely related to pathogenicity (Liang et al. 2010; Kim et al. 2008). This has also proven to be the case in plant-pathogenic bacteria, including *X. campestris* pv. *campestris* (Dow et al. 2003). In the current study, both the $\Delta ihfA$ and $\Delta ihfB$ mutant strains showed a significant reduction in motility and biofilm formation compared with the wild-type strain EC1. Expression analyses showed that the expression of important flagellum gene *fliC* was significantly decreased in the IHF mutants. Bacterial flagella are known to play important roles in swimming motility, biofilm formation, and virulence (Chua et al. 2003). Therefore, we suspect that bacterial motility plays an essential role in the initial stages of *D. zea* infection and indicates that IHF positively regulates bacterial motility and biofilm formation.

Extracellular enzymes have been well-characterized in *Pectobacterium* and *Dickeya* species because of their essential roles in the development of soft rot symptoms (Matsumoto et al. 2003). Some pathogenic bacteria produce cell wall-degrading enzymes, such as pectinases, polygalacturonases, proteases, and cellulases, as dominant virulent factors. These enzymes help to acquire nutrients by breaking down the cell walls of plants and aid in pathogenicity (Eriksson et al. 1998; Reverchon et al. 2016). In our study, compared with the wild-type, protease activity of the IHF mutants decreased by >82%, while the activity of pectinases was decreased by 62%. qRT-PCR analysis showed that the expressions of *pelA*, *pelB*, *pelC*, *pehX*, and *prtG* was significantly reduced in strains $\Delta ihfA$ and $\Delta ihfB$, which agrees with the results of the phenotypic assays. It is interesting to note that, although we did not observe any significant differences in cellulase activity between the mutant and the wild-type strains in the plate assays, qRT-PCR analysis showed a significant decrease in the expression of *celY* and *celZ* in $\Delta ihfB$ and a significant increase in *celY* expression in $\Delta ihfA$ compared with the wild-type strain. Cellulases are enzyme complexes containing several different enzymatic subunits, including endocellulases, exocellulases, cellobiases, oxidative cellulases, and cellulose phosphorylases. As we only examined two of the genes involved in these complexes (*celY* and *celZ*), it is perhaps not surprising that the cellulase enzyme activity shown by the plate test was not reflected in the changes in expression of the limited number of genes examined. Further, changes in gene expression do not necessarily directly translate to higher or lower enzyme activity.

In some pathogenic bacteria, including *Vibrio cholerae* (Stonehouse et al. 2008), *Pseudomonas syringae* pv. *phaseolicola* (Arvizu-Gómez et al. 2011), and *D. zea* (Zhou et al. 2011), toxins are a vital virulence factor. In *Pseudomonas syringae* pv. *phaseolicola*, IHF binds to the promoter region of the *phtD* operon, which is involved in phaseolotoxin synthesis (Arvizu-Gómez et al. 2011). Zeamine toxin is a key virulence factor of *D. zea* in both rice seeds and plants (Zhou et al. 2011). Our results indicated that $\Delta ihfA$ and $\Delta ihfB$ produced little zeamine toxin and showed a significantly decreased inhibitory effect on rice seed germination and reduced development of soft rot in potato and rice plants. Further qRT-PCR analysis verified that the expression of *zmsA*, the zeamine toxin synthesis gene in *D. zea* (Zhou et al. 2011), was four times lower in the $\Delta ihfA$ and $\Delta ihfB$ mutants than in the wild type, accounting for the reduced toxin activities of the mutant strains.

Therefore, we propose that IHF is required for the production of zeamine toxin in *D. zea*.

Type 3 secretion systems (T3SS), encoded by the *hrp* gene cluster, are essential virulence factors in many plant-pathogenic bacteria and play a role in eliciting the HR in plants (Deane et al. 2010; Lara-Tejero et al. 2011; Yap et al. 2005). Our results indicated that IHF mutants $\Delta ihfA$ and $\Delta ihfB$ did not cause a HR in tobacco, and our qRT-PCR analysis revealed significant decreases in the expression of *hrpN* and *hrpY* (the important gene in *Hrp* cluster) in the IHF mutants. In *Erwinia amylovora*, the regulatory protein HrpY forms a complex with HrpS that binds to the promoter region of *hrpL*, thereby inducing gene expression (Wei et al. 2000). However, recent studies have shown that HrpY binds to the promoter of HrpS to regulate HrpS expression (Lee and Zhao 2018). Previously, we found that the HrpX/HrpY two-component system of *D. zea* positively regulates bacterial motility, biofilm formation, and the expression of downstream *hrp* genes and, also, plays a role in the early stages of infection of rice plants (Chen et al. 2014). Taken together, these results imply that IHF may regulate the T3SS of *D. zea*.

Cyclic di-GMP is a second messenger for many bacteria, but its synthesis and degradation require two key enzymes, DGC and PDE (Schirmer and Jenal 2009). Extensive research has delineated the important role of bacterial c-di-GMP in regulating biofilm formation and the expression of virulence genes (Chin et al. 2010; Dahlstrom et al. 2016; Jenal et al. 2017; Römmling et al. 2013; Ryan 2013). In *Dickeya dadantii*, two phosphodiesterases, EcpB and EcpC, which are involved in the synthesis and degradation of c-di-GMP, could adjust and positively control the expression of the T3SS, thereby influencing cellular behaviors as well as virulence. However, deletion of one or both *ecpB* and *ecpC* enhanced biofilm formation but repressed swimming and swarming motility and reduced pectate lyase production (Yi et al. 2010). Our results showed that IHF positively regulates the expression of DGC, a key enzyme

Fig. 5. Effects of *Dickeya zea* wild-type strain EC1, $\Delta ihfA$, and $\Delta ihfB$, and their complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$, on the rice seed germination. **A**, Rice seeds were immersed in bacterial supernatants for 5 h, were washed three times with sterilized water, and were then incubated for 4 days at 30°C, to observe the germination of rice seeds. **B**, The length of the rice plumule and root were measured. Luria Bertani medium was used as a blank control (CK). The values are means of three replicates and the error bars are standard deviation. Statistical analysis was performed on each group of data and significantly different values (analysis of variance $P < 0.05$) are indicated by different letters.

in the synthesis of cyclic di-GMP, and is positively associated with motility, biofilm formation, toxin production, and enzyme activity. These findings agree with a previous study that showed that deletion of DGC protein CgsA was associated with a reduction of virulence in *Xylella fastidiosa* (Chatterjee et al. 2010). However, in *Xanthomonas oryzae* pv. *oryzae*, DgcA, a DGC, negatively affected virulence, exopolysaccharides

production, bacterial autoaggregation, and motility, but positively affected biofilm formation via modulation of the intracellular c-di-GMP levels (Su et al. 2016). These findings indicate that the roles and functions of DGC proteins can vary in different plant-pathogenic bacteria. In addition, bacterial c-di-GMP levels are affected by the key enzymes DGC and PDE. Further investigation is needed to determine the role of

Fig. 6. Virulence assay of *Dickeya zeae* wild-type strain EC1, $\Delta ihfA$, $\Delta ihfB$, and their complementation strains, R- $\Delta ihfA$ and R- $\Delta ihfB$, on potato, rice, and tobacco. **A**, Symptoms of soft rot in potato. Healthy potato tube pieces were inoculated with 2 μ l of bacterial suspensions (optical density at 600 nm [OD₆₀₀] = 0.5) and were incubated at 30°C for 24 h, to observe soft rot on the potato piece. The arrows indicate inoculation positions. **B**, Symptoms of soft rot on rice plants. The bacterial suspensions (OD₆₀₀ = 0.5) were injected into the base of rice seedlings, and the curling and wilt of interior leaves, watery brown spots in the leaf sheath at the stem base, were observed 2 days later. **C**, Hypersensitive response on tobacco leaves. Bacterial suspensions (OD₆₀₀ = 0.5) were injected into the tobacco leaves and, 24 h later, the hypersensitive response on the inoculation sites was observed.

c-di-GMP in *D. zeeae*, although the present results show that IhfA binds to the promoter region of *DGC* in *D. zeeae*, thereby controlling the expression of *DGC*.

Bacterial genomes are likely to contain multiple target gene promoters containing the conserved IHF binding sequence. For example, IHF binds to the promoter sequence of the *virB* operon (the type IV secretion system) in *Brucella abortus* to regulate the transcription of downstream genes and alter bacterial virulence (Sieira et al. 2004). In *Shigella flexneri*, IHF positively regulates virulence gene expression by binding to multiple promoters, including those associated with *virF*, *virB*, and *icsA* (Porter and Dorman 1997). In other plant pathogens, IHF binds to the promoters of *hrpL* in *Pectobacterium carotovorum* subsp. *carotovorum* (Chatterjee et al. 2002), *hrpS* in *Pantoea stewartii* subsp. *stewartii* (Merighi et al. 2006), and the *phtD* operon, which is involved in phaeolotoxin synthesis, in *Pseudomonas syringae* pv. *phaseolicola* (Arvizu-Gómez et al. 2011). Our results showed that IhfA binds to the promoter region of the *DGC* operon in *D. zeeae*, which is involved in the synthesis of c-di-GMP, to alter bacterial behaviors. These results suggest that IHF is involved in the regulation of many different target genes in different pathogenic bacteria.

In summary, we demonstrated that IhfA binds to the promoter region of *DGC* in *D. zeeae*, which may affect the concentration of the second bacterial messenger, c-di-GMP, thereby influencing bacterial motility, biofilm formation, and the production of extracellular enzymes and toxins. Further, deletion of the IHF-encoding genes *ihfA* and *ihfB* resulted in reduced expression of several virulence factors, including the T3SS and its virulence effector proteins. Therefore, we suggest that IHF is an important integrated regulator of pathogenicity in *D. zeeae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and the plasmids used in this study are outlined in Table 1. All bacterial strains of *D. zeeae* were grown on minimal medium (MM) (per liter: 10.5 g of K_2HPO_4 , 4.5 g of KH_2PO_4 , 2.0 g of mannitol, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 0.005 g of $FeSO_4$, 0.002 g of $MnCl_2$, 2 ml of glycerol, 15 g of agar, pH 7.0) at 30°C, while *E. coli* strains were cultured in LB medium at 37°C. Rifampicin (50 µg/ml), streptomycin (50 µg/ml), kanamycin (50 µg/ml), and ampicillin (100 µg/ml), all purchased from Sigma, were used when appropriate.

Biochemical reagents, primers, and other materials.

Restriction enzymes *SpeI*, *BamHI*, *HindIII*, *EcoRI*, D2000 DNA ladder marker, PrimerSTARTTM HS DNA polymerase, *Taq* DNA polymerase, T4 DNA ligase, Star Prep plasmid miniprep kit, Universal DNA purification kit, DNA fragment purification kit, PrimeScript RT master mix (for real time) and SYBR Premix Ex *Taq* II (Tli RNaseH Plus) were purchased from TaKaRa. SV total RNA isolation system was purchased from Promega. Primer synthesis and sequencing were performed by Invitrogen. Primer sequences are shown in Table 2.

Gene knockout and complementation of IHF-encoding genes in *D. zeeae*.

Deletion mutants $\Delta ihfA$ and $\Delta ihfB$ were generated from the wild-type *D. zeeae* strain EC1 by homologous recombination (Chen et al. 2014). Briefly, left and right homologous arms of *ihfA* were amplified by PCR, using genomic DNA from *D. zeeae* EC1 as template and primer sets *ihfA*-U-F/*ihfA*-U-R and *ihfA*-D-F/*ihfA*-D-R, respectively. The two amplified

Fig. 7. Relative expression of genes associated with the virulence of *Dickeya zeeae* wild-type strain EC1, $\Delta ihfA$, and $\Delta ihfB$ by quantitative reverse-transcription polymerase chain reaction at 24 h of growth. Gene of 16S rDNA was used as an endogenous control to normalize gene expression data. Target genes were *zmsA* (encodes a novel multimodular polyketide synthase for zeamine toxin), Hrp system (*hrpN* and *hrpY*), pectate lyase genes (*pelA*, *pelB*, *pelC*), polygalacturonases (*pehX*), *celY*, and *celZ* (cellulase), *prtG* (serralysin G), *fliC* (a regulatory gene in the flagellum system), and *DGC* (diguanylate cyclase). The values are means of three replicates and the error bars are standard deviation.

fragments, which flanked *ihfA*, were fused together by PCR, using primers *ihfA*-U-F and *ihfA*-D-R. Following digestion with *Spe*I and *Bam*HI, the fusion fragments were purified and were ligated into suicide vector pKNG101, to obtain recombinant plasmid pKNG- Δ *ihfA*. The recombinant plasmid was then transformed into *E. coli* K12CC118 and the transformed bacteria were grown on LB plates with streptomycin (50 µg/ml) at 37°C for 24 h. Positive clones were detected by PCR using primers pKNG101-F/R and were verified by sequencing. The recombinant *E. coli* strain carrying plasmid pKNG- Δ *ihfA* and helper plasmid pRK2013 and wild-type strain EC1 were separately cultured overnight and were then mixed together for approximately 10 h. During mixed culture, helper plasmid pRK2013 facilitated the transfer of the recombinant plasmid into the receptor *D. zeae* strain (triparental mating). As the recombinant plasmid contained sequences flanking the target gene that were homologous to the sequences in the *D. zeae* EC1 strain, homologous double-crossover recombination by the suicide vector resulted in deletion of the target gene. PCR analysis was used to confirm the deletion of *ihfA* in the Δ *ihfA* mutant strain. A Δ *ihfB* mutant strain was constructed using the same method.

For complementation of Δ *ihfA* and Δ *ihfB*, the promoter and coding sequences of the *ihfA* and *ihfB* genes were amplified from *D. zeae* EC1 genomic DNA. The resulting amplicons were ligated into vector pBBR1MCS4. The recombinant plasmids, designated pBBR1-*ihfA* and pBBR1-*ihfB*, were transformed into *E. coli* DH5 α . The resulting transformants were selected on LB plates containing ampicillin and streptomycin and were confirmed by PCR. Triparental mating was then performed

between the recombinant plasmid, the mutant strain, and the pRK2013 helper plasmid, and the candidate complementation strains were screened using antibiotic selection. The resulting complementation strains, R- Δ *ihfA* and R- Δ *ihfB*, were confirmed by plasmid extraction, double digestion of the isolated plasmid, and sequencing.

Bacterial growth curve.

To examine the growth of bacterial strains, cells from overnight cultures of the tested strains were harvested and were inoculated into fresh LB broth. The strains were then cultured at 30°C with shaking at 200 rpm. Every 2 h, 2-ml volumes of each culture were collected to measure the optical density at 600 nm (OD₆₀₀). Three replicates were performed for each strain, and bacterial growth curves were plotted based on the average values.

Motility and biofilm-formation assays.

Determination of cell motility for each strain was conducted by measuring the diameters of the colonies on plates, as described previously (Shen et al. 2001; Yap et al. 2005). Briefly, all strains were cultured in MM overnight to achieve an OD₆₀₀ = 1.5. Aliquots (2 µl) of bacterial suspensions were then inoculated onto the surfaces of agar plates (10 g of tryptone, 5 g of NaCl, and 2.5g of agar per liter) and were incubated at 30°C for 24 h. Each assay was repeated three times in triplicate.

The ability of the strains to form a biofilm was examined as described previously (Shen et al. 2001). A 10-µl aliquot of bacterial cell culture (OD₆₀₀ = 0.5) was inoculated into tubes containing SONG medium (per liter: 20 g of tryptone, 5 g of yeast extract, 5g of NaCl, 2.4 g of MgSO₄·7H₂O, 0.186 g of KCl, 50 ml of 40% glycerin) and was incubated for 72 h at 28°C.

Examination of extracellular enzyme and toxin production.

Bacterial protease, pectinase, and cellulase production was measured using protease, pectinase, and cellulase test plates,

Fig. 8. Promoter binding region of *ihfA* protein to target gene *DGC* (diguanylate cyclase) and electrophoretic mobility shift assay. **A**, Schematic of the intergenic region between transcriptional regulator and DGC in which the integration host factor (IHF) binding-site position is represented with a barrel. The alignment of the DGC IHF binding site with the consensus IHF binding site (Arvizu-Gómez et al. 2011) is also shown. W, A or T; R, A or G; N, any base. **B**, Electrophoretic mobility shift assay for testing the binding of *ihfA* protein to DGC promoter. Arrows indicate the free probe (the bottom bands) and the protein-DNA complex (the top band).

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics ^a	Origin
<i>Dickeya zeae</i> EC1	Wild type, Rif ^R	Lab collection
Δ <i>ihfA</i>	<i>ihfA</i> knockout mutant of EC1	This study
R- Δ <i>ihfA</i>	Complementation strain of Δ <i>ihfA</i>	This study
Δ <i>ihfB</i>	<i>ihfB</i> knockout mutant of EC1	This study
R- Δ <i>ihfB</i>	Complementation strain of Δ <i>ihfB</i>	This study
<i>Escherichia coli</i> K12 CC118	<i>gyrA</i> , <i>recA</i> , λ <i>pir</i>	Lab collection
DH5 α	<i>deoR</i> , <i>recA</i> , <i>endA</i> , <i>hdsR</i> , <i>supE</i> , <i>thi</i> , <i>gyrA</i> , <i>relA</i>	Lab collection
Plasmids		
pKNG101	Sm ^R , <i>SacB</i> , <i>mobRK2</i> , <i>oriR6K</i> (pir-minus)	Lab collection
pBBR1MCS4	Amp ^R , Mob ⁺ , Tra ⁻ , Cosmetic	Lab collection
pRK2013	Tra ⁺ , Mob ⁻ , ColE1-replicon, Kan ^R , Sp ^R	Lab collection
pKNG- Δ <i>ihfA</i>	<i>ihfA</i> knockout fragment ligated on pKNG101	This study
pBBR1- <i>ihfA</i>	<i>ihfA</i> complementation fragment ligated on pBBR1MCS4	This study
pKNG- Δ <i>ihfB</i>	<i>ihfB</i> knockout fragment ligated on pKNG101	This study
pBBR1- <i>ihfB</i>	<i>ihfB</i> complementation fragment ligated on pBBR1MCS4	This study

^a Rif^R, Sm^R, Amp^R, Kan^R, and Sp^R = resistant to rifampicin, streptomycin, ampicillin, kanamycin, and spectinomycin.

respectively, as described by Chatterjee et al. (1995). Aliquots of overnight cultures grown in MM were inoculated onto test plates and were further cultivated at 30°C for 24 h. Each assay was repeated three times in triplicate. The protease test plates were observed directly, while the pectinase test plates were immersed in 1-mol/liter HCl to allow observation of the transparent zone. The cellulase test plates were stained with 0.1% (wt/vol) Congo red for 30 min and were then washed twice with 1 M NaCl for 15 min each time.

D. zea produces a toxin that demonstrates bactericidal activity against *E. coli*. LB agar plates spread with a 1 in 10 dilution of an overnight culture of *E. coli* DH5 α were used for the toxin assay (Zhou et al. 2011). Aliquots of each of the test bacterial cultures were then spotted onto the plates. Bacterial inhibition was observed following culture at 30°C for 24 h. Each assay was repeated three times in triplicate.

Rice seed germination assay.

A rice seed germination assay was conducted as described by Zhou et al. (2011). Briefly, 50 healthy rice seeds were immersed in 25 ml of bacterial suspension for 5 h and were then washed three times with sterile water. The cleaned seeds were placed on moistened filter paper in a petri dish and were then incubated at 30°C with a 16-h light and 8-h dark cycle. LB

broth was used as a control. The lengths of the root and bud of the germinated seeds were measured following 4 days of incubation.

Potato tubers soft rot assay.

A potato tuber soft rot assay was carried out as described previously (Zhou et al. 2011). Healthy potato tubers were washed and were sliced into pieces with a thickness of 5 mm. The surface of each slice was air dried on an ultra-clean bench and was then exposed to ultraviolet light for 15 min. Each slice was then placed in a petri dish lined with filter paper moistened with sterilized water. The surface of each slice was pricked with a sterilized needle and 2- μ l aliquots of the bacterial suspensions (strains were cultured in LB medium to OD₆₀₀ = 0.5) were inoculated into the holes made by the needle. LB broth was used as a negative control. The inoculated potato slices were then incubated at 30°C for 24 h. Each assay was repeated three times in triplicate. Potato slices were monitored regularly for the appearance of soft rot symptoms.

Assays for HR on tobacco leaves and virulence in rice plants.

Wild-type *D. zea* strain EC1 along with the knockout mutants and complementation strains were individually inoculated

Table 2. Primers used in this study^a

Primer	Sequences (5' to 3')	Size(bp)	Applications
ihfA-U-F	CGGGATCCGTGTTTGAGAGCGGTTTACG	690	Fragment flanking the left of <i>ihfA</i> gene
ihfA-U-R	CAGGACTCTTTGGGTGACGCCATAGTTAATCCCTCAAGG		
ihfA-D-F	CCTTGAGGGATTAACCTATGGCGTCACCCAAAGAGTCTCTG	432	Fragment flanking the right of <i>ihfA</i> gene
ihfA-D-R	CGGACTAGTTATGAGCGTGCCTTTCCAGC		
ihfA-F	CCCAAGCTTGGAAATTGCCGCTACCGTTG	377	Complementation fragment of <i>ihfA</i>
ihfA-R	CGGGATCCATCAGGACTCTTTGGGTGAC		
ihfB-U-F	CGGGATCCCGTAGTGGAAGTTATGGTTC	771	Fragment flanking the left of <i>ihfB</i> gene
ihfB-U-R	CAGGCGTTATGACTGACTAAGTGGTCAGTAAATACCACG		
ihfB-D-F	CGTGGTATTTACTGACCACTTAGTCAGTCATAACGCCTG	472	Fragment flanking the right of <i>ihfB</i> gene
ihfB-D-R	CGGACTAGTGGACTACAACAGGAACAGGT		
ihfB-F	CCCAAGCTTAAGGCGAGTAATGACGGTGG	443	Complementation fragment of <i>ihfB</i>
ihfB-R	CGGGATCCGACAAAAATAGCACCCGGC		
ihfA-CX-F	CGGTGAGCAGGCATTGATTC	1399	For <i>ihfA</i> deletion sequencing
ihfA-CX-R	AGCACCTAAACGGCGACAG		
ihfB-CX-F	CGAAGAAGGCGTTGAAGGTC	1482	For <i>ihfB</i> deletion sequencing
ihfB-CX-R	CGTTCTGACCACTTCTCCAGC		
16S-qF	GCAACGCGAAGAACCTTACCT	94	For qRT-PCR
16S-qR	GCCATGCAGCACCTGTCT		
zmsA-qF	CCGGGCTCGTTTGATTTCTTTTG	81	For qRT-PCR
zmsA-qR	CGCTTTGTCTGAACGCAACATC		
hrpN-qF	AGGGCGATCAGAAGTCTTTGC	119	For qRT-PCR
hrpN-qR	CGCCTGGCCTAAACCATTACC		
hrpY-qF	ATCGTGTGTCGGGATTGTC	70	For qRT-PCR
hrpY-qR	TGCTCGCGTGTGGTCAAC		
pelA-qF	CCACCAAAGATGGCGAAACCT	86	For qRT-PCR
pelA-qR	GCTGTTGGAGATGGTCACGTAGT		
pelB-qF	CGTCACCATCTCCTACAACCTACATC	78	For qRT-PCR
pelB-qR	TCAGCGGTGTCAGAAGAACTG		
pelC-qF	CAACCGCTATAACGACGTGAAC	99	For qRT-PCR
pelC-qR	AGACCGGAACCTGGTGACATTG		
celY-qF	CGTCGGATGGTGATGTACTGAT	90	For qRT-PCR
celY-qR	TGACCGATGACCGCTTTCTG		
celZ-qF	AGCGATGGCTGAATCTGAATCC	100	For qRT-PCR
celZ-qR	GCACTGTTGCTGTCAATGTCG		
prtG-qF	TGGGATGGTCAAGCGAATCAG	93	For qRT-PCR
prtG-qR	CAACGCCGTACCGACAATCTTC		
pehX-qF	CGGTCGTAAGGGTAGCCATAC	78	For qRT-PCR
pehX-qR	GCAGGCCGATGTCGCTTT		
fliC-qF	GGTGCAGACTGCTGAAGGT	90	For qRT-PCR
fliC-qR	CGGTCAAGAGAACCGTTGGTATC	90	For qRT-PCR
DGC-qF	GAAGATTCGTCAGGCCGTTTC	90	For qRT-PCR
DGC-qR	ATTAGATTCCGCAACACCGC		
MCS-F	TCTTCGCTATTACGCCAGCT		Universal primer of pBBR1MCS4
MCS-R	GGCTCGTATGTTGTGTGGAA		

^a Underlined sequences indicate the restriction enzyme cleavage sites. qRT-PCR = quantitative reverse-transcription polymerase chain reaction.

into LB broth supplemented with the appropriate antibiotics. Bacterial cells were collected by centrifugation at 6,000 rpm for 10 min and were resuspended in sterile water to an OD₆₀₀ = 0.5. The bacterial suspensions were injected into tobacco leaves and onto the base of rice seedlings (three pots per treatment, three seedlings per pot), using a sterilized needle-free injector. LB broth was used as a negative control. Symptoms of HR were observed on the tobacco leaves within 24 h of inoculation. Rice seedlings were monitored regularly for symptom development.

Expression analysis of some important virulent genes.

To verify the genes regulated by IHF, the expression of several virulence genes was examined by qRT-PCR analysis. Total bacterial RNA was extracted using a SV total RNA isolation system kit, and the eluted RNA was DNase-treated using Turbo DNA-free, according to the manufacturer's instructions (Promega). The quality and quantity of the total RNA were assessed using agarose gel electrophoresis and spectrophotometer, respectively. Reverse transcription for cDNA synthesis was performed using PrimeScript RT master mix (TaKaRa), and 100 ng of cDNA was used for qRT-PCR analysis using a SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TaKaRa). The 16S rRNA gene was used as an endogenous control to normalize gene-expression data. Target genes *zmsA* (encodes a novel multimodular polyketide synthase for zeamine toxin), *hrpN* and *hrpY* (Hrp system), pectate lyase genes (*pelA*, *pelB*, and *pelC*), polygalacturonases (*pehX*), cellulase genes (*celY* and *celZ*), *prtG* (Serralyisin G), *fliC* (a regulatory gene in flagellum system), and *DGC* were analyzed. Relative expression levels of the target genes were calculated using the 2^{-ΔΔCt} method (Livak et al. 2001), with the following formulas.

$$\text{Relative expression levels of target gene} = 2^{-\Delta\Delta C_t} \quad (1)$$

$$-\Delta\Delta C_t = -(\Delta C_{tq} - \Delta C_{tcb}) \quad (2)$$

where Ct is the threshold cycle, q is the treated sample, and cb is the control sample.

Binding of IhfA to the DGC promoter.

Expression and purification of IhfA. An *E. coli* codon-optimized synthetic *D. zeae ihfA* gene for protein expression was purchased from Invitrogen. Forward (5'-CATATGCAC CATCACCATCACCAT-3') and reverse (5'-AAGCTTTCA TTAACCTTTCTTTTCGGGC-3') primers were then designed to amplify *ihfA*. The amplified *ihfA* gene was cloned into protein expression vector pET30a at the *NdeI* and *HindIII* restriction sites, generating plasmid pET30a-ihfA. The recombinant plasmid was then transformed into *E. coli* BL21(DE3). The resulting transformant strain was cultured and protein expression was induced with isopropyl β-D-thiogalactopyranoside. Expressed protein IhfA was then purified from *E. coli* BL21 (DE3) cells by affinity chromatography (Ni-IDA).

EMSA. The synthesis and labeling of a DGC probe with biotin were performed by Invitrogen. The probe sequence (TAGTTATTAATCTTTATTTATCAGTTGATTACGTTGATA AATTCGGTCC) was derived from the DGC promoter region. The EMSA binding reaction mixtures (10 μl) contained EMSA/gel-shift binding buffer (5×), purified *D. zeae* IhfA, and DGC probe. Control reactions without one or both IhfA and DGC probe were included. Reactions were incubated at room temperature for 20 min and were then loaded into a 6% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. Following electrophoresis, the resolved proteins with bound probes were transferred to a positively charged nylon membrane. After UV crosslinking, chemiluminescence detection was carried out.

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