The integration host factor regulates multiple virulence pathways in bacterial pathogen Dickeya zeae MS2

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Abstract

Dickeya zeae is an aggressive bacterial phytopathogen that infects a wide range of host plants. It has been reported that integration host factor (IHF), a nucleoid-associated protein consisting of IHFα and IHFβ subunits, regulates gene expression by influencing nucleoid structure and DNA bending. To define the role of IHF in the pathogenesis of D. zeae MS2, we deleted either and both of the IHF subunit encoding genes ihfA and ihfB, which significantly reduced the production of cell wall-degrading enzymes (CWDEs), an unknown novel phytotoxin and the virulence factor-modulating (VFM) quorum-sensing (QS) signal, cell motility, biofilm formation, and thereafter the infection ability towards both potato slices and banana seedlings. To characterize the regulatory pathways of IHF protein associated with virulence, IHF binding sites (consensus sequence 5′-WATCAANNNNTTR-3′) were predicted and 272 binding sites were found throughout the genome. The expression of 110 tested genes was affected by IHF. Electrophoretic mobility shift assay (EMSA) showed direct interaction of IhfA protein with the promoters of vfmE, speA, pipR, fis, slyA, prtD, hrpL, hcb, hcp, indA, hdaA, flhD, pilT, gcpJ, arcA, arcB, and lysR. This study clarified the contribution of IHF in the pathogenic process of D. zeae by controlling the production of VFM and putrescine QS signals, phytotoxin, and indigoidine, the luxR-solo system, Fis, SlyA, and FlhD transcriptional regulators, and secretion systems from type I to type VI. Characterization of the regulatory networks of IHF in D. zeae provides a target for prevention and control of plant soft rot disease.

KEYWORDS

cell motility, cell wall-degrading enzymes, Dickeya zeae, DNA binding, integration host factor, transcriptional regulator, virulence

Shanshan Chen and Ming Hu contributed equally to this work.

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1 | INTRODUCTION

Dickeya zeae, formerly named Erwinia chrysanthemi pv. zeae, is responsible for widespread outbreaks of banana bacterial soft rot disease (Samson et al., 2005; Zhang et al., 2013). The disease develops upwards from the base of the pseudostem to the growing point and causes soft rot in plant tissues with symptoms of leaf wilting and yellowing (Lin et al., 2010; Zhang et al., 2014). D. zeae strains isolated from different hosts, or even the same host species, have different degrees of virulence (Hu et al., 2018). The pathogen uses complex pathogenic mechanisms, mainly arising from the diversity of the virulence factors and pathogenic regulatory networks. Currently, four classes of pathogenic regulatory factors have been unveiled in D. zeae: a quorum-sensing (QS) system, cyclic-di GMP (c-di-GMP) signalling, two-component signal transduction systems (TCS), and transcription factors (TF) (Chen et al., 2016; Hommais et al., 2008; Lv et al., 2019; Reverchon et al., 1998). These regulatory factors coordinate to modulate pathogen virulence when induced by a variety of environmental factors.

Nucleoid-associated proteins (NAPs), which are chromosome-organizing factors, affect the transcriptional landscape of bacterial cells (Dey et al., 2017). Integration host factor (IHF), which is composed of two subunits IHFα and IHFβ, is a small heterodimer of NAPs (Reverchon et al., 2021). It can recognize specific DNA sequences and bind to the small groove of the DNA to form an IHF–DNA complex, resulting in DNA helix reversals over short distances (Craig & Nash, 1984; Richet et al., 1986). IHF is able to specifically interact with phage attachment sites (attP) to regulate the expression of phage λ (Craig & Nash, 1984; Richet et al., 1986). IHF to positively regulates the early and repressor transcription of phage Mu by a type of binding site dependence (van Rijn et al., 1989). Genetic and biochemical studies have shown that IHF is involved in physiological processes of Escherichia coli, in which a conserved sequence of IHF binding sites in λ phage attP is also found within functional regions (Craig & Nash, 1984; Friedman, 1988). In E. coli, IHF binding to DNA depends on the consensus sequence 5′-WATCAANNNNTTR-3′ (where W = A or T, N = any base, and R = A or G) (Craig & Nash, 1984; Yang & Nash, 1989). This consensus sequence is useful for predicting potential sites for IHF binding to DNA from other pathogenic bacteria. In Vibrio cholerae and Vibrio fluvialis, VgrG/Tsl and its downstream effector and immune genes act as the tip component of the type VI secretion system (T6SS), and are positively regulated by the quorum-sensing (QS) regulator HapR and the global regulator IHF. IHF appears to be much more activated than HapR, suggesting that IHF may be a major regulator of the vgrG operon (Zhang et al., 2021). IHF activates the transcription of GbdR, a specific regulator of choline metabolism in Pseudomonas aeruginosa (German Sanchez et al., 2017). IHF also significantly regulates the expression of RpoN-dependent hrlP and other type III secretion system (T3SS) genes, and is essential for Erwinia amylovora virulence. In addition, it regulates swimming motility through activation of sRNA rsmB expression (Lee & Zhao, 2016). In Pseudomonas putida, IHF promotes the frequency of homologous recombination and point mutations (Mikkel et al., 2020). Notably, Fis has been identified as a global regulator that has complex interactions with IHF, as reflected in the ability of both to identify consensus cis-elements and consensus binding sites (Oliveira Monteiro et al., 2020). Recently, it was found that IhfA is able to bind to the promoter region of a diguanylate cyclase-encoding gene to alter the c-di-GMP expression level and regulate pathogenicity as well as other physiological functions in Dickeya oryzae (Chen et al., 2019).

Numerous studies have confirmed the important role of IHF in bacteria, including affecting gene expression, controlling cell metabolism, and inducing DNA bending (Priede et al., 2012; Reverchon et al., 2021; van Rijsewijk et al., 2011). However, the regulatory mechanism of IHF in D. zeae is unclear, and in particular how IHF directly interacts with downstream genes associated with virulence is still unknown. This study aims to clarify the specific pathways directly regulated by IHF to affect the virulence of D. zeae.

2 | RESULTS

2.1 | Deletion of IHF slightly affects the growth of D. zeae MS2

To study the role of IHF in the pathogenicity of D. zeae MS2, single knockout mutants, ΔihfA and ΔihfB, and a double knockout mutant, ΔihfAB, were first generated by homologous recombination. Subsequently, complemented strains of ΔihfA and ΔihfB were constructed by in trans expression of genes into the corresponding mutants using a low-copy expression vector pLAFR3, respectively named as ΔihfA::ihfA and ΔihfB::iHFb. To facilitate the growth rate of MS2 and its derivatives, optical density values at 600 nm within 48 h were recorded using Bioscreen C. As shown in Figure S1, ΔihfA, ΔihfB, and ΔihfAB grew slightly slower than MS2 and the complemented strains. However, the growth of the complemented strains was greater than that of MS2 at 12–48 h postincubation. These results showed that the deletion of IHF slightly affects the growth of D. zeae MS2, which is similar to the situation in Dickeya dadantii 3937 and D. oryzae EC1 (Chen et al., 2019; Reverchon et al., 2021).

2.2 | IHF is a global transcriptional regulator modulating the expression of over 100 genes in D. zeae MS2

Because IHF is known to modulate multiple phenotypes associated with virulence in many pathogenic bacteria, to identify the regulatory pathways of IHF associated with virulence in D. zeae we first searched for the IHF binding motif (5′-WATCAANNNNTTR-3′) throughout the MS2 genome. The results revealed a total of 272 candidates with IHF binding sites in the promoter regions of D. zeae MS2. Among them, 101 genes were found to be upregulated and 71 genes to be downregulated in ΔihfA. Notably, 35 genes were significantly differentially expressed in both ΔihfA and ΔihfB, and 30 genes in ΔihfAB. This indicates that IHF plays a key role in regulating the expression of these genes.

In conclusion, the study provides new insights into the role of IHF in the pathogenicity of D. zeae MS2. Further research is needed to investigate the specific pathways directly regulated by IHF to affect the virulence of D. zeae.
coding sequences (CDS) in the MS2 genome (Table S1). To verify whether the promoters interact with IHF, reverse transcription-quantitative PCR (RT-qPCR) was performed to analyse the expression of the genes downstream of 128 randomly selected promoters from the 272 candidates in the ihf mutants in comparison to the wild type (Table 1). According to the RT-qPCR results, the expression of 110 genes was significantly regulated by IHF, 105 positively and 5 negatively (Table 1). The predicted functions of the regulated genes indicated that IHF could regulate a variety of physiological, stress resistance, and pathogenic phenotypes of D. zeae MS2, for instance metabolic processes including the transport of nutrients, cations, and amino acids, signal transduction, secretion systems, production of secondary metabolites and cell wall-degrading enzymes (CWDEs), and capsular polysaccharide synthesis (Table 1).

2.3 | IHF positively regulates the virulence factor-modulating and putrescine QS systems

The virulence of Dickeya spp. is controlled by highly sophisticated regulatory networks, a crucial one of which is the regulation by QS systems including the N-acylhomoserine lactone (AHL) system encoded by expIR (Feng et al., 2019; Hussain et al., 2008), the virulence factor-modulating (VFM) system encoded by the vfm gene cluster (Lv et al., 2019; Nasser et al., 2013), and the putrescine system, encoded by speA and speC (Liu et al., 2022; Shi et al., 2019). Furthermore, a luxR-solo system has also been characterized in D. zeae MS2, which regulates the activity of proline iminopeptidase PipA and contributes to the virulence of MS2 by an unknown mechanism (Feng et al., 2019). To determine whether IHF regulates QS systems in D. zeae MS2, RT-qPCR analysis of the expression of specific QS systems was performed. The results showed that deletion of ihfA, ihfB, and ihfAB significantly decreased the expression of vfmE, speA, speC, pipR, and pipA, but not the expression of expI and expR (Table 1). To determine whether IHF could directly interact with these genes, an electrophoretic mobility shift assay (EMSA) was performed to test the binding ability of IHF to their promoters containing the IHF motif. The results showed that 2.5 μM of IhfA protein could bind to the promoter regions of vfmE, speA, and pipR but not speC (Figure 1). In addition, the production of AHL and VFM QS signals was also measured using the biosensors previously described (Hussain et al., 2008; Lv et al., 2019). The results indicated that deletion of the IHF proteins did not affect the production of the AHL signal (Figure 2a), but dramatically blocked the production of the VFM signal (Figure 2b).

2.4 | IHF positively regulates the production of CWDEs via multiple regulatory pathways

CWDEs, including pectinases, cellulases, and proteases that degrade the structure of host plant cells, are important pathogenicity factors in Dickeya spp. and cause soft rot symptoms (Hugouvieux-Cotte-Pattat et al., 1996). Semiquantitative measurement of the enzymatic activities showed that deletion of ihf significantly reduced the activities of cellulases, pectate lyases, polygalacturonases, and proteases (Figure 3a).

In-depth study of virulence factors in Dickeya pathogens has revealed multiple complex regulatory pathways controlling the production of CWDEs. The first one is the VFM QS system regulating the generation of CWDEs (Lv et al., 2019; Nasser et al., 2013). Because IhfA directly binds to the promoter of vfmE (Figure 1), the decreased production of the VFM signal (Figure 2b) is one of the causes of the reduced production of CWDEs in the ihf mutants. Many transcriptional regulators are known to participate in the regulation of CWDE production, such as PecS, PecT, H-NS, SlyA, Fis, and KdgR (Hommais et al., 2008; Lv et al., 2018; Nasser & Reverchon, 2002; Rodionov et al., 2004; Zhou et al., 2016). IHF significantly regulated the expression of fis, slyA, pecT, and two H-NS genes hnsA and hnsB (Table 1). The EMSA results also confirmed the direct interaction of IhfA with fis and slyA promoters (Figure 1).

2.5 | IHF positively regulates the expression of type I to type VI secretion systems

Secretion systems, such as the type I secretion system (T1SS) and the type II secretion system (T2SS), responsible for the secretion of CWDEs, might be influenced by IHF proteins. Therefore, the expression of the genes related to secretion systems that harboured the predicted IHF binding motif was tested in the ihf mutants. Among the regulated genes, prtD expression was significantly reduced (2.34–3.27 log₂ fold change) in the ΔihfA, ΔihfB, and ΔihfAB mutants compared with that in wild-type MS2 (Table 1). The EMSA results also confirmed the direct interaction of IhfA with the prtD promoter (Figure 1). prtD is the first gene of the T1SS operon prtDEF, responsible for the secretion of proteases (Lory, 1998; Palacios et al., 2001). The reduced expression of prtD in the mutants probably results in less secretion of proteases and smaller protease degradative halos (Figure 3a). The expression of the gspC gene, encoding the inner membrane protein GspC of T2SS, decreased by 2.45–4.06 log₂ fold change in the ihf mutants (Table 1). The T2SS is devoted to the secretion of most extracellular pectinases and cellulases that promote host invasion (Login et al., 2010; Wang et al., 2012), reasonably explaining the reasons for the decreased enzymatic activities of pectinases and cellulases of the ihf mutants (Figure 3a). The lack of binding to the gspC probe suggests an indirect regulation of IHF on T2SS expression (Figure 1).

The type III secretion system (T3SS) encoded by the dsp/hrp/hrc gene clusters in D. zeae MS2 has been studied recently and also contributes to the host range and virulence of D. zeae (Hu et al., 2022). In the T3SS, HrpL is the master regulator controlling the expression of the whole system and the effectors (T3SEs) in Dickeya, Erwinia, and
| Function category                  | Gene       | Locus (CI030) | Product                                      | Binding site (5′-WATCAANNNNTTR-3) | Position from start codon | Log₂ fold change of gene expression† |
|-----------------------------------|------------|---------------|----------------------------------------------|----------------------------------|---------------------------|--------------------------------------|
| **Signal transduction**           | vfmE       | RS00505       | AraC family transcriptional regulator        | AATCAAACCAGTA                    | −99 to −73                | −3.01 ± 0.16 ↓ −5.01 ± 0.09 ↓ −6.21 ± 0.13 ↓ |
|                                  | expR       | RS00610       | LuxR family transcriptional regulator        | AATTAATAGCTA                     | −234 to −208              | 0.01 ± 0.05 −0.10 ± 0.09 −0.18 ± 0.07 − |
|                                  | expl       | RS00615       | Acyl-homoserine-lactone synthase             | AATCAACGATAA                      | −100 to −74               | 0.08 ± 0.14 −0.29 ± 0.27 −0.56 ± 0.14 − |
|                                  | /          | RS05855       | TonB-dependent siderophore receptor          | TATCAACAATTTT                    | −69 to −43                | −4.32 ± 0.23 ↓ −4.01 ± 0.06 ↓ −4.22 ± 0.41 ↓ |
|                                  | citA       | RS09785       | Sensor histidine kinase regulating citrate/malate metabolism | AATCAAATGATG                     | −309 to −283              | 0.05 ± 0.08 −3.11 ± 0.13 ↓ −1.49 ± 0.16 ↓ |
|                                  | narX       | RS11960       | Nitrate/nitrite two-component system sensor histidine kinase NarX | CATCAACAGCTTG                    | −377 to −351              | −1.64 ± 0.14 −0.74 ± 0.30 −0.91 ± 0.10 − |
|                                  | narL       | RS11965       | Two-component system response regulator NarL | AATCAACGGCTCG                    | −390 to −364              | −3.15 ± 0.14 −0.66 ± 0.12 −1.73 ± 0.19 ↓ |
|                                  | pipR       | RS14530       | LuxR-solo transcriptional regulator          | TATCAACGCGTA                     | 547 to −521               | −4.13 ± 0.62 −4.99 ± 0.53 −3.42 ± 0.44 ↓ |
|                                  | pipA       | RS14535       | Proline iminopeptidase-family hydrolase      | No box                           | −4.94 ± 0.28              | −5.42 ± 0.50 −3.12 ± 0.12 − |
|                                  | speA       | RS18265       | Arginine decarboxylase                       | AATCAATAATCA                      | −766 to −739              | −3.17 ± 0.36 −2.47 ± 0.12 −3.81 ± 0.32 ↓ |
|                                  | speC       | RS04655       | Ornithine decarboxylase                      | No box                           | −0.70 ± 0.12              | −1.61 ± 0.59 −2.25 ± 0.34 ↓ |
|                                  | pmrB       | RS18960       | Acid pH activated two-component system sensor histidine kinase PmrB | AATCAACAGGTGTA                   | −268 to −242              | −2.19 ± 0.33 −3.78 ± 0.14 −2.90 ± 0.08 ↓ |
| **Cell wall-degrading enzymes**   | inh        | RS10815       | Apr/l family metalloprotease inhibitor Inh   | TATCAATGCTTT                      | −84 to −58                | −1.78 ± 1.31 −2.27 ± 1.29 −1.54 ± 0.54 ↓ |
|                                  | peLE       | RS15350       | Polysaccharide lyase PeLE                   | AATCAACTAGTT                      | −269 to −243              | −0.04 ± 0.13 −0.22 ± 0.22 −0.46 ± 0.32 − |
| **Type I secretion system**       | prenty     | RS10810       | Type I secretion permease/ATPase Prenty       | TATCAATGCTTT                      | −463 to −437              | −2.47 ± 0.20 −3.27 ± 0.16 −2.34 ± 0.54 ↓ |
| **Type II secretion system**      | gspC       | RS14420       | Type II secretion system protein GspC        | CATCAACGCCGTA                    | −723 to −697              | −2.63 ± 0.08 −3.52 ± 0.54 −2.53 ± 0.08 ↓ |
| **Type III secretion system**     | hrPL       | RS11655       | RNA polymerase σ factor HrPL                 | TAACATGTGTTA                    | −234 to −208              | −2.26 ± 0.04 −4.34 ± 0.27 −2.68 ± 0.03 ↓ |
| **Type IV secretion system**      | rhs        | RS03620       | Type IV secretion protein Rhs                | CATCAATGCTTTA                    | +455 to +482              | −2.98 ± 0.22 −4.85 ± 0.06 −2.65 ± 0.11 ↓ |
|                                  |           | RS06845       | Type IV secretion protein Rhs                | CATCAATCGGTA                     | −865 to −839              | −3.45 ± 0.30 −4.01 ± 0.10 −5.28 ± 0.15 ↓ |
| **Type V secretion system**       | hecB       | RS11555       | HecB hemolysin secretion protein HecB        | AATCAAGGCTTG                     | −150 to −124              | −3.22 ± 0.07 −4.37 ± 0.30 −1.84 ± 0.36 ↓ |
| **Type VI secretion system**      | hcp        | RS03605       | Hcp family type VI secretion protein         | AATCAAGATTTTC                    | −883 to −857              | −5.05 ± 0.11 −7.02 ± 0.23 −5.01 ± 0.08 ↓ |
| **Secondary metabolite synthesis**| indA       | RS00465       | Indigoidine synthase IndA                    | TATTAATGCTTA                     | −268 to −241              | −1.71 ± 0.13 −4.62 ± 0.13 −3.96 ± 0.55 ↓ |
|                                  | hdaA       | RS05020       | Hypothetical protein                         | AAACAAAAATTTC                   | −224 to −197              | −3.22 ± 0.14 −3.11 ± 0.16 −4.25 ± 0.14 ↓ |
|                                  | hdaL       | RS05075       | Nonribosomal peptide synthetase              | No box                          | −4.29 ± 0.44              | −5.37 ± 0.22 −5.97 ± 0.24 ↓ |
|                                  | fldA        | RS06215       | Flavodoxin FldA                               | AATCAACTAATGG                    | −35 to −9                 | 0.56 ± 0.08 −0.10 ± 0.09 −0.20 ± 0.20 − |
TABLE 1 (Continued)

| Function category          | Gene     | Locus (C1O30) | Product                        | Binding site (5'-WATCAANNNNTTR-3) | Position from start codon | Log₂ fold change of gene expression b |
|----------------------------|----------|---------------|--------------------------------|------------------------------------|---------------------------|-------------------------------------|
| Motility and chemotaxis    | mcp      | RS02500       | Methyl-accepting chemotaxis protein | ATCAATAATTG                       | -180 to -154              | -2.27 ± 0.06 ↓ | -3.62 ± 0.18 ↓ | -2.38 ± 0.24 ↓ |
|                           | mcp      | RS02880       | Methyl-accepting chemotaxis protein | TATCAATAAGTTA                     | -262 to -236              | -3.45 ± 0.01 ↓ | -4.84 ± 0.39 ↓ | -3.21 ± 0.62 ↓ |
|                           | pilV     | RS08665       | Shufflon system plasmid conjugative transfer pilus tip adhesin PIIV | TATCAATGTTTT                     | -131 to -105              | -3.40 ± 0.13 ↓ | -5.03 ± 0.16 ↓ | -3.18 ± 0.22 ↓ |
|                           | ompX     | RS09065       | Outer membrane protein OmpX      | AATCAAAAAATTA                     | -243 to -217              | -1.51 ± 0.07 ↓ | -0.87 ± 0.06 - | -0.85 ± 0.04 - |
|                           | mcp      | RS09390       | Methyl-accepting chemotaxis protein | TATCACCAGGTTG                     | -626 to -599              | 1.30 ± 0.34 ↑ | 1.08 ± 1.22 ↑ | 1.53 ± 1.06 ↑ |
|                           | mcp      | RS12040       | HAMP domain-containing protein   | CATCAATAATTG                      | -494 to -468              | 1.28 ± 0.21 ↑ | 0.09 ± 0.06 - | 0.96 ± 0.05 - |
|                           | cheA     | RS13530       | Chemotaxis protein CheA          | TATCAAAATTTT                      | +5 to +31                 | 1.49 ± 0.10 ↑ | 1.36 ± 0.21 ↑ | 0.01 ± 0.02 - |
|                           | flhD     | RS13550       | Flagellar transcriptional regulator FlhD | TATCAATGTATTA                    | -485 to -459              | -1.28 ± 0.14 ↓ | -2.51 ± 0.62 ↓ | -1.24 ± 0.08 ↓ |
|                           | pilV     | RS14140       | HAMP domain-containing protein   | TATCAATGTATTA                    | -445 to -419              | -0.77 ± 0.08 - | -0.97 ± 0.04 - | -0.94 ± 0.05 - |
|                           | pilV     | RS17065       | Type IV pilus twitching motility protein PilV | TATCACCCTTTC                    | -252 to -226              | -4.17 ± 0.43 ↓ | -5.42 ± 0.32 ↓ | -3.31 ± 0.12 ↓ |
|                           | mcp      | RS20725       | Methyl-accepting chemotaxis protein | AATCAAGGCGGTG                     | -580 to -554              | -1.27 ± 0.18 ↓ | -2.44 ± 0.18 ↓ | -1.77 ± 0.16 ↓ |
| c-di-GMP                   | gcpl     | RS06990       | Sensor domain-containing diguanylate cyclase DGC | TATCAAGCAGTATTA                  | -761 to -735              | -1.49 ± 0.10 ↓ | -3.30 ± 0.18 ↓ | -1.72 ± 0.22 ↓ |
|                           | gcpJ     | RS14540       | Diguanylate cyclase DGC          | TATCAAGGCTATTA                    | -479 to -453              | -3.46 ± 0.34 ↓ | -5.82 ± 0.26 ↓ | -4.34 ± 0.43 ↓ |
|                           | gcpA     | RS15425       | Sensor domain-containing diguanylate cyclase DGC | TATCAAAATATAT                    | -315 to -289              | 1.77 ± 0.06 ↑ | 1.43 ± 0.18 ↑ | 0.25 ± 0.46 - |
| Capsular polysaccharide synthesis | wcaJ  | RS02585       | Undecaprenyl-phosphate glucose phosphotransferase WcaJ | TATCAATCAATTT                    | +54 to +80                | -3.34 ± 0.07 ↓ | -5.28 ± 0.10 ↓ | -4.45 ± 0.11 ↓ |
|                           | cpsD     | RS02600       | Polysaccharide biosynthesis tyrosine autokinase CpsD | CATCAACTGATTT                    | -97 to -71                | -4.02 ± 0.58 ↓ | -6.00 ± 0.59 ↓ | -5.61 ± 0.08 ↓ |
| /                          | RS02605   | Capsular biosynthesis protein   | TATCAAGCAGTATTA                  | -300 to -274                    | -3.10 ± 0.14 ↓ | -4.81 ± 0.12 ↓ | -3.87 ± 0.37 ↓ |
| /                          | RS02615   | Glycosyltransferase            | CATCAACCTTTA                     | -8 to +16                      | -4.07 ± 0.25 ↓ | -5.79 ± 0.09 ↓ | -4.84 ± 0.26 ↓ |
| wza                       | RS06590  | Polysaccharide export protein Wza | CATCAACGTTATTA                   | -131 to -105                    | -2.14 ± 0.36 ↓ | -5.02 ± 0.60 ↓ | -3.39 ± 0.17 ↓ |
| wcaA                      | RS06605  | Glycosyltransferase family 4 protein WcaA | TATCAATCCTTA                    | +1 to +27                      | -2.81 ± 0.31 ↓ | -2.02 ± 0.21 ↓ | -2.53 ± 0.33 ↓ |
|                           | RS06620  | Glycosyltransferase family 2 protein | AATCAAGCGTTG                     | -561 to -535                    | -3.30 ± 0.03 ↓ | -3.97 ± 0.14 ↓ | -2.97 ± 0.19 ↓ |
| /                          | RS06690  | Glycosyltransferase            | AATCAAGCGTTG                     | -222 to -196                    | -3.84 ± 0.32 ↓ | -2.54 ± 0.04 ↓ | -2.07 ± 0.06 ↓ |
### TABLE 1 (Continued)

| Function category | Gene | Locus (CLO30) | Product | Binding site (5′-WATCAANNNNTTR-3′)* | Position from start codon | Log₂ fold change of gene expression |
|-------------------|------|---------------|---------|-------------------------------------|---------------------------|-----------------------------------|
| **Stress resistance** |      |               |         |                                     |                           |                                   |
| arcB              | RS01695 | Aerobic respiration two-component sensor histidine kinase ArcB | ATTACGGCTGTG | -6 to +20 | $-1.30 \pm 0.32$ | $-1.28 \pm 0.47$ | $-1.31 \pm 0.34$ |
| /                 | RS08685 | DHA2 family efflux MFS transporter permease subunit | TATCA ATAGATT | -161 to -135 | $-3.64 \pm 0.31$ | $-4.13 \pm 0.11$ | $-2.70 \pm 0.07$ |
| /                 | RS09235 | Bcr/CfiA family multidrug efflux MFS transporter | AATCCGGCTTG | -133 to -107 | $-3.36 \pm 0.09$ | $-3.89 \pm 0.36$ | $-3.72 \pm 0.16$ |
| rhlA              | RS09440 | Biosurfactant synthesis, colonization of plant surface, alpha/beta hydrolase | TATCA AACAGTT | -502 to -476 | $-3.92 \pm 0.30$ | $-4.06 \pm 0.14$ | $-3.64 \pm 0.24$ |
| symE              | RS11525 | Type I toxin-antitoxin system SymE family toxin | CATCA ACGGTG | -565 to -539 | $-1.94 \pm 0.29$ | $-4.48 \pm 0.36$ | $-2.69 \pm 0.14$ |
| yafN              | RS16150 | Type I toxin-antitoxin system antitoxin YafN | AATCA TTTTTT | -107 to -81 | $-1.97 \pm 0.10$ | $-2.54 \pm 0.03$ | $-1.40 \pm 0.20$ |
| proV              | RS16475 | Glycine betaine/L-proline ABC transporter ATP-binding protein ProV | AATCACGGT | -253 to -227 | $-1.04 \pm 0.04$ | $-2.17 \pm 0.04$ | $-1.61 \pm 0.22$ |
| pspG              | RS17225 | Envelope stress response protein PspG | AATCAATGGTTA | -138 to -112 | $-2.25 \pm 0.37$ | $-4.69 \pm 0.30$ | $-3.14 \pm 0.21$ |
| robA              | RS18160 | MDR efflux pump AcrAB transcriptional activator RobA | AATCA ATTCAT | -129 to -103 | $-1.51 \pm 0.11$ | $-1.24 \pm 0.03$ | $-1.84 \pm 0.36$ |
| **Metabolic process** |      |               |         |                                     |                           |                                   |
| xylH              | RS00425 | Sugar ABC transporter permease XylH | GATCAATCGT | -193 to -167 | $-2.74 \pm 0.08$ | $-4.16 \pm 0.04$ | $-2.72 \pm 0.17$ |
| elbB              | RS01700 | Isopenoid biosynthesis glycoxaldehyde ElbB | AATCA ATTAGT | -52 to -26 | $0.11 \pm 0.02$ | $-0.40 \pm 0.10$ | $-1.35 \pm 0.05$ |
| /                 | RS02510 | GNAT family N-acetyltransferase | TATCA AGCCTT | -82 to -56 | $-3.15 \pm 0.16$ | $-4.30 \pm 0.08$ | $-4.72 \pm 0.22$ |
| fueO              | RS03185 | Lactatedehyde reductase FucO | AATCA ATACAT | -410 to -15 | $-0.38 \pm 0.24$ | $-0.77 \pm 0.15$ | $-1.44 \pm 0.17$ |
| actP              | RS03835 | Cation/acetate symporter ActP | AATCA ACGAT | -307 to -281 | $-1.19 \pm 0.06$ | $-1.68 \pm 0.05$ | $-2.85 \pm 0.14$ |
| pheB              | RS05035 | Phosphate response regulator transcription factor PhoB | TATCA GCGGT | +65 to +91 | $-3.26 \pm 0.37$ | $-3.68 \pm 0.23$ | $-2.37 \pm 0.05$ |
| kdpF              | RS06280 | K⁺-transporting ATPase subunit F | CATCA ATAGTTA | -336 to -310 | $-2.97 \pm 0.15$ | $-3.64 \pm 0.21$ | $-1.90 \pm 0.30$ |
| /                 | RS07470 | SDR family oxidoreductase | AATCA AATATT | -165 to -139 | $-2.29 \pm 0.37$ | $-4.48 \pm 0.04$ | $-3.31 \pm 0.11$ |
| hlyD              | RS08935 | Secretion protein HlyD | AATCA ATAGTTA | -258 to -232 | $0.17 \pm 0.09$ | $-0.02 \pm 0.20$ | $0.35 \pm 0.09$ |
| deor              | RS08995 | DeoR/GlipR transcriptional regulator | TATCA ATCTGT | -182 to -156 | $-1.50 \pm 0.03$ | $-1.58 \pm 0.32$ | $-2.18 \pm 0.20$ |
| dsbB              | RS11160 | Disulphide bond formation protein DsbB | TATCA ATAGT | -770 to -744 | $-6.45 \pm 0.24$ | $-1.95 \pm 0.18$ | $-1.51 \pm 0.10$ |
| lcpP              | RS12295 | L-lactate permease LcpP | TATCA ATAGTTA | -50 to -24 | $1.97 \pm 0.09$ | $0.31 \pm 0.13$ | $0.80 \pm 0.09$ |
| ptsG              | RS13040 | PTS sugar transporter subunit IIC PtSG | TATCA ACATT | -173 to -147 | $-1.43 \pm 0.07$ | $-3.20 \pm 0.12$ | $-1.80 \pm 0.16$ |
| fabG              | RS13300 | SDR family oxidoreductase FabG | AATCA ATTTTT | -238 to -212 | $-1.10 \pm 0.16$ | $1.62 \pm 0.07$ | $0.31 \pm 0.25$ |
| /                 | RS13660 | Glycosyltransferase family 1 protein | TATCA ACTCTA | -199 to -173 | $-3.62 \pm 0.18$ | $-4.26 \pm 0.36$ | $-3.55 \pm 0.03$ |
| gmhA              | RS16340 | D-sedoheptulose 7-phosphate isomerase GmhA | TATCA ATCTTT | -285 to -259 | $0.02 \pm 0.14$ | $-0.85 \pm 0.21$ | $-0.28 \pm 0.26$ |
| /                 | RS16795 | D-galactonate transporter MFS | TATCA ATGTTA | -697 to -671 | $-1.81 \pm 0.15$ | $-1.32 \pm 0.06$ | $-1.59 \pm 0.35$ |
| /                 | RS17250 | Transporter substrate-binding domain-containing protein | AATCA ACGAGTTA | -125 to -99 | $-4.17 \pm 0.37$ | $-2.13 \pm 0.31$ | $-2.52 \pm 0.22$ |
| aceE              | RS18640 | Malate synthase AceA | AATCA ATAGTTA | -177 to -151 | $-0.93 \pm 0.11$ | $-1.62 \pm 0.03$ | $-1.21 \pm 0.21$ |
| /                 | RS20745 | NAD(P)/FAD-dependent oxidoreductase | AATCA ATGCA | -180 to -154 | $-1.85 \pm 0.03$ | $-3.89 \pm 0.10$ | $-3.01 \pm 0.13$ |
| Function category | Gene       | Locus (C1O30) | Product                                                                 | Binding site (5′-WATCAANNNNTTR-3′) | Position from start codon | Log₂ fold change of gene expression |
|-------------------|------------|---------------|-------------------------------------------------------------------------|-------------------------------------|---------------------------|-----------------------------------|
| Amino acid transport | RS08960   | ABC transporter substrate-binding protein | AAATCAAGGCTTC | −75 to −49 | −2.07 ± 0.13 | ↓ −2.67 ± 0.12 | ↓ −1.85 ± 0.29 |
|                   | RS09455   | Aspartate:alanine antiporter | TATCAATAATTTA | −52 to −26 | −1.97 ± 0.23 | ↓ −2.42 ± 0.35 | ↓ −1.92 ± 0.16 |
|                   | RS13315   | DegT/DnrJ/EryC1/StS family aminotransferase | AAATCATATATTAT | +68 to −34 | 1.09 ± 0.24 | ↑ 1.41 ± 0.06 | ↑ 1.07 ± 0.54 |
| Replication, recombination, and repair | cos3      | CRISPR-associated helicase/endonuclease Cas3 | AAATCAACATGGTGG | −366 to −340 | 0.56 ± 0.17 | − 0.37 ± 0.18 | − 0.80 ± 0.07 |
|                   | fxSA       | Bacteriophage exclusion-mediated protein FxsA | TATCAATGAGTTA | −510 to −25 | −1.02 ± 0.14 | ↓ −1.96 ± 0.16 | ↓ −2.33 ± 0.12 |
|                   | decoC      | Deoxyribosyl-phosphate aldolase DeoC | TATCAATAGTTA | −940 to −414 | −1.01 ± 0.08 | ↓ −1.84 ± 0.21 | ↓ −1.61 ± 0.08 |
|                   | dcm        | DNA cytosine methyltransferase Dcm | AAATCATATTATA | −360 to −334 | −1.10 ± 0.08 | ↓ −1.40 ± 0.06 | ↓ −3.63 ± 0.02 |
|                   | rof        | Rho-binding antiterminator | TATCAAACATTATA | +5 to +31 | 2.38 ± 0.15 | ↓ −2.18 ± 0.28 | ↑ −3.22 ± 0.42 |
|                   | cas3       | DNA-directed RNA polymerase subunit β | TATCAATTGTTA | −137 to −111 | −2.07 ± 0.09 | 0.53 ± 0.03 | −2.32 ± 0.21 |
|                   | /          | Integrate arm-type DNA-binding domain-containing protein | AAATCAATGTTA | −148 to −122 | −2.83 ± 0.23 | ↓ −3.72 ± 0.13 | ↓ −3.41 ± 0.04 |
| Transcriptional regulators | cspE       | Transcription antiterminator/RNA stability regulator CspE | AAATCAATGTTA | −361 to −335 | 1.60 ± 0.19 | ↑ 1.49 ± 0.18 | ↑ 2.16 ± 0.21 |
|                   | ttcA       | tRNA 2-thiocytidine(32) synthetase TtcA | AAATCAATGTTA | −50 to +21 | −3.07 ± 0.29 | ↓ −1.75 ± 0.13 | ↓ −2.47 ± 0.32 |
|                   | /          | Site-specific integrase | AAATCAATGTTA | −118 to −82 | −0.26 ± 0.09 | −1.45 ± 0.13 | −1.26 ± 0.05 |
|                   | bxC        | BREX phase resistance system P-loop protein Bxc | AAATCAAGGTTTAT | −262 to −236 | −5.06 ± 0.47 | ↓ −3.29 ± 0.40 | ↓ −3.24 ± 0.47 |
|                   | mtrR       | MtrR family transcriptional regulator | GAATCACGCTTTG | −404 to −378 | −3.56 ± 0.14 | ↓ −4.95 ± 0.17 | ↓ −4.80 ± 0.06 |
|                   | fis        | DNA-binding transcriptional regulator Fis | AAATCAACCCATA | −108 to −81 | −4.03 ± 0.22 | −4.90 ± 0.08 | −4.31 ± 0.25 |
|                   | shyA       | MarR family transcriptional regulator | TATCAATCACTTG | −124 to −98 | −2.70 ± 0.06 | −4.17 ± 0.39 | −2.15 ± 0.11 |
|                   | /          | LuxR C-terminal-related transcriptional regulator | AAATCAAGGTTTAT | −360 to −114 | −3.06 ± 0.11 | −5.01 ± 0.47 | −3.48 ± 0.20 |
|                   | /          | HoxR family transcriptional regulator | AAATCAAGGTTTAT | −483 to −457 | −2.60 ± 0.37 | −4.48 ± 0.36 | −4.01 ± 0.10 |
|                   | /          | LysR family transcriptional regulator | AAATCAAGGTTTAT | −269 to −243 | −1.19 ± 0.06 | −1.68 ± 0.05 | −2.85 ± 0.14 |
|                   | flhA       | Formate hydrogenlyase transcriptional activator FlhA | GAATCAAGGTTTAT | −107 to −81 | −0.61 ± 0.05 | −0.86 ± 0.04 | −0.61 ± 0.16 |
|                   | hnsA       | H-NS histone family protein | AAATCAAGGTTTAT | −328 to −302 | −2.45 ± 0.09 | −3.58 ± 0.27 | −2.00 ± 0.26 |
|                   | hnsB       | H-NS histone family protein | AAATCAAGGTTTAT | −154 to −128 | −2.02 ± 0.09 | −2.83 ± 0.08 | −2.78 ± 0.22 |
|                   | arsR       | Metalloregulator ArsR/SmtB family transcription factor | TATCAATTCCTTA | −181 to −155 | −3.83 ± 0.08 | −3.88 ± 0.38 | −4.48 ± 0.14 |
|                   | lrp        | Leucine-responsive transcriptional regulator Lrp | AAATCAATGTTA | −549 to −523 | −2.84 ± 0.08 | −0.16 ± 0.11 | −1.41 ± 0.35 |
|                   | /          | Rok family transcriptional regulator | AAATCAATGTTA | −121 to −95 | 0.36 ± 0.24 | −1.25 ± 0.01 | 0.05 ± 0.31 |
|                   | lysR       | LysR family transcriptional regulator | AAATCAATGTTA | −312 to −286 | −3.31 ± 0.15 | −4.74 ± 0.29 | −3.55 ± 0.15 |
|                   | pecT       | LysR family transcriptional regulator | AAATCAATGTTA | −62 to −36 | −2.60 ± 0.19 | −3.85 ± 0.21 | −1.99 ± 0.35 |
|                   | mngR       | GntR family transcriptional regulator MngR | TATCAATGTTTAT | −320 to −240 | −5.04 ± 0.13 | −4.65 ± 0.28 | −3.48 ± 0.39 |
|                   | /          | MarR family transcriptional regulator | AAATCAATGTTA | −106 to −80 | −1.35 ± 0.26 | −1.26 ± 0.20 | −1.92 ± 0.06 |

*Continues*
| Function category | Gene (C1O30) | Locus | Product | Binding site (5′-WATCAANNNNTTR-3′) | Position from start codon | Log₂ fold change of gene expression b | ΔihfA | ΔihfB | ΔihfAB |
|-------------------|--------------|-------|---------|-----------------------------------|---------------------------|--------------------------------------|-------|-------|-------|
| Hypothetical proteins | / | RS00200 | DUF3861 domain-containing protein | TATCAACATTTTG | −108 to −82 | −2.88 ± 0.10 ↓ | −4.46 ± 0.03 ↓ | −3.47 ± 0.13 ↓ |
| / | RS01690 | TIGR01212 family radical SAM protein | AATCAATATGTTT | +4 to +30 | −2.86 ± 0.13 ↓ | −2.87 ± 0.16 ↓ | −3.00 ± 0.23 ↓ |
| / | RS02900 | Hypothetical protein | AATCAAAATATTA | −341 to −315 | −3.61 ± 0.24 ↓ | −4.14 ± 0.24 ↓ | −2.99 ± 0.01 ↓ |
| / | RS03390 | DUF485 domain-containing protein | TATCAACGATTTG | +2 to +28 | −1.10 ± 0.08 ↓ | −1.40 ± 0.06 ↓ | −3.63 ± 0.02 ↓ |
| / | RS03400 | Hypothetical protein | TATCAACGTTTTG | −151 to −125 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS03585 | Hypothetical protein | AATCAACGGATTG | +2 to +28 | −1.10 ± 0.08 ↓ | −1.40 ± 0.06 ↓ | −3.63 ± 0.02 ↓ |
| / | RS03590 | Hypothetical protein | AATCAACGGGTTC | +30 to +56 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS04495 | IGR00645 family protein | AATCAATGTGTTA | −109 to −83 | −1.62 ± 0.46 ↓ | −2.51 ± 0.05 ↓ | −1.93 ± 0.25 ↓ |
| / | RS05170 | Hypothetical protein | TATCAATATCTTG | +32 to +58 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS06855 | Hypothetical protein | AATCAATATCTTG | +32 to +58 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS06880 | Hypothetical protein | GATCAATTCTTA | −120 to −14 | −6.34 ± 0.62 ↓ | −4.26 ± 0.95 ↓ | −4.49 ± 0.17 ↓ |
| / | RS07745 | DUF4160 domain-containing protein | AATCAAATGATTG | −20 to +6 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS08605 | Hypothetical protein | AATCAAATGATTG | −20 to +6 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS09035 | DUF1349 domain-containing protein | AATCAAATGATTG | −20 to +6 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS10060 | Hypothetical protein | AATCAAATGATTG | −20 to +6 | −2.86 ± 0.13 ↓ | −3.00 ± 0.23 ↓ | −2.82 ± 0.28 ↓ |
| / | RS10540 | YolA family protein | TATCAACGTTTTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS10730 | Hypothetical protein | AATCAAATGATTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS10850 | Hypothetical protein | AATCAAATGATTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS11855 | DUF1852 domain-containing protein | TATCAATATCTTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS12735 | Hypothetical protein | TATCAATATCTTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS13635 | Hypothetical protein | TATCAATATCTTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |
| / | RS14145 | Hypothetical protein | TATCAACGTTTTG | −316 to −290 | −2.58 ± 0.14 ↓ | −3.15 ± 0.14 ↓ | −3.16 ± 0.14 ↓ |

Notes: Genes with statistically significant changes in expression (|log₂ fold change of expression| ≥ 1 and p ≤ 0.05) were selected.

a Italics represent the mismatch of binding sites.

b ↓ represents down-regulation of gene expression, ↑ represents up-regulation of gene expression, – represents no significant change in gene expression. Data are the results of at least three independent biological replicates and are means ± SD.
Pseudomonas (Hu et al., 2022; Waite et al., 2017). IHF is required for hrpL gene expression in E. amylovora (Lee & Zhao, 2016). In our study, a similar regulation pattern of hrpL was observed with 2.22–4.61 log2 fold change reduction of expression in the ihf mutants (Table 1). EMSA indicated that IhfA, at a concentration of 2.5 μM, directly interacted with the promoter of hrpL (Figure 1).

The D. zeae MS2 genome lacks most of the type IV secretion system (T4SS) coding genes, only retaining virB1 (C1O30_RS07565), virB2 (C1O30_RS07560), and two Rhs-encoding genes (C1O30_RS03620 and C1O30_RS06845). The expression of these two rhs genes was positively regulated by IHF through the IHF motif located at 455–482 bp in the C1O30_RS03620 open reading frame (ORF) and 865–839 bp upstream of the C1O30_RS06845 ORF (Table 1).

Type V and type VI secretion systems (T5SS and T6SS) are both involved in contact-dependent competition (Koskiniemi et al., 2013). The former consists of two partner proteins, the HecB/TspB outer membrane protein allowing the secretion of the large HecA/TspA protein (Aoki et al., 2010). IHF positively regulated the expression of hecB by 1.48–4.67 log2 fold change (Table 1) and IhfA directly interacted with the promoter of hecB (Figure 1). In Dickeya spp., the T6SS comprises the secretion machinery encoded by the imp/vas operon, the haemolysin-coregulated protein (Hcp) and the valine-glycine repeat protein G (VgrG) that form a membrane puncturing device, and the Rhs protein effectors (Zhou et al., 2015). Deletion of ihf genes dramatically decreased the expression of hcp by 4.93–7.25 log2 fold change (Table 1). EMSA demonstrated the direct control of IhfA on hcp (Figure 1).

2.6 | IHF positively regulates the production of indigoidine and a novel phytotoxin

Prediction of the IHF binding motif in the MS2 genome also identified the conserved binding site in the promoter of indA (−268 to −242 bp upstream of the start codon). indA is the first gene of the indABC operon responsible for the biosynthesis of indigoidine, which is essential for resistance to oxidative stress (Reverchon et al., 2002). RT-qPCR analysis showed that deletion of ihf genes significantly decreased the expression of indA (Table 1). EMSA also verified the interaction of IhfA with the promoter of indA (Figure 1).

Apart from indigoidine, D. zeae MS2 also produces another uncharacterized secondary metabolite that inhibits the growth of Escherichia coli and many pathogenic fungi (Feng et al., 2019; Hu et al., 2018). The biosynthetic gene cluster has been preliminarily predicted by antiSMASH and the importance of C1O30_RS05075 in the production of this phytotoxin has been determined (Feng et al., 2019).
et al., 2019). Analysis of the organization of this gene cluster revealed that genes from C1O30_RS05020 to C1O30_RS05100 comprise the operon. We named these genes hdaA–hdaQ. Interestingly, an IHF binding motif was predicted in the promoter sequence of hdaA (C1O30_RS05020) (Table 1). To verify the regulation of IHF on this secondary metabolite, RT-qPCR was first performed for the expression of hdaA and the previously identified hdaL (C1O30_RS05075). The results showed that the expression of both hdaA and hdaL was greatly reduced, by 3.11–5.97 log₂ fold change, in the ihf mutants compared with wild-type MS2 (Table 1). EMSA also confirmed a direct interaction of IhfA with hdaA (Figure 1). The production of the phytotoxin was then

![Image](image-url)
2.7 | Mutation of *ihf* reduces cell motility and biofilm formation

Surface motility, biofilm formation, and host invasion are some of the manifestations of functional responses to surface colonization (Harshey, 2003). Biofilm formation is important for bacteria to survive in a harsh environment and attach to the host surface, while bacterial motility is one of the major determinants for pathogen diffusion and invasion. In this study, we tested whether cell motility and biofilm formation were affected by IHF. As shown in Figure 4a, all the *inf* mutants (∆*ihfA*, ∆*ihfB*, and ∆*ihfAB*) exhibited an obvious decrease in swimming, swarming, and twitching motility; complementation of *ihfA* and *ihfB* into the corresponding mutants partially recovered cell motility. In addition, the nonadherent and attached biofilms formed by the deletion mutants were much less than wild-type MS2 and the complemented strains (Figures 4b and 5c).
Motility and chemotaxis are essential for bacteria when searching for favourable niches to invade plants. During this process, flagella-mediated movement is vital and under the control of multiple regulatory systems. FlhDC is the master regulator of flagellar genes controlling cell motility, CWDE expression, and the T3SS in *D. dadantii* 3937 (Yuan et al., 2015). In this study, the expression of *flhD* was 1.24–2.51 log₂ fold change decreased in the *ihf* mutants (Table 1). Moreover, 4 μM of IhfA bound to the promoter of *flhD* (Figure 1), suggesting direct control of IHF on flagella-mediated motility. Furthermore, *pilT*, which encodes type IV pili that contribute to twitching motility, was decreased in expression by over 3.31 log₂ fold change in the *ihf* mutants (Table 1); EMSA also verified a direct interaction of IhfA with *pilT* at a concentration of 2.5 μM (Figure 1).

Cyclic-di-GMP (c-di-GMP) is a second messenger chemical in bacteria, playing an important role in cell division, motility, and virulence regulation. The turnover of c-di-GMP in bacterial cells is effected by its synthesis by diguanylate cyclases (DGCs) and degradation by phosphodiesterases (PDEs). DGC catalyses the formation of c-di-GMP through a conserved GGDEF domain (Tal et al., 1998). Previous investigations have characterized the repertoires of c-di-GMP turnover genes in the *D. dadantii* 3937 and *D. oryzae* EC1 genomes (Chen et al., 2016; Yi et al., 2010). Like the EC1 strain, the genome of *D. zae* MS2 harbours 12 GGDEF domain proteins, three EAL domain proteins, and three GGDEF-EAL hybrid proteins (Chen et al., 2016). Among the genes encoding these proteins, only three, C1O30_RS06990 (gcpl, with dCache_1 and GGDEF domains), C1O30_RS14540 (gcpl, with TM, GGDEF, and d2mhr_ domains), and C1O30_RS15425 (gcpA, with PAS, PAC, GAF and GGDEF domains), have the IHF binding motif in their promoter regions (Table 1). RT-qPCR analysis showed that the expression of *gcpl* and *gcpJ* was significantly reduced in the *ihf* mutants (Table 1). In *D. oryzae* EC1, IHF protein has been reported to bind to the promoter region of a DGC protein (Chen et al., 2019) that is a homologue of GcpJ. EMSA confirmed the direct binding of IhfA to the promoter of *gcpJ* (Figure 1).

## 2.8 | IHF mediates capsular polysaccharide synthesisCPS and stress resistance of *D. zae* MS2

Capsular polysaccharide is an essential virulence factor for pathogenesis of bacteria and plays a role in protecting bacteria against...
phagocytosis and environmental stresses. The biosynthesis of capsular polysaccharides involves multiple genes. In strain MS2, eight genes related to the biosynthesis of capsular polysaccharides were identified as containing the IHF binding motif and all of them were positively regulated by IHF (Table 1). Toxin-antitoxin operons are ubiquitous in bacteria and their products can cause cell growth arrest (Zhang et al., 2012). Expression of yafN and symE, which encode two toxin-antitoxin proteins, was activated by IHF (Table 1). Furthermore, a multidrug resistance (MDR) efflux pump AcrAB transcriptional activator, RobA, was also identified as positively regulated by IHF because its expression was reduced in the \( \text{ihf} \) mutants (Table 1). RobA has been reported to positively regulate the most important MDR efflux pump in \( E. coli \) (Tanaka et al., 1997). Altogether, the above results suggest the possibility of a deficiency of stress resistance in \( \text{ihf} \) mutants.

### 2.9 IHF mediates the metabolic process of \( D. zeae \) MS2

A significant decrease in \( \text{ihf} \) mutants of the expression of genes involved in the metabolic pathway that achieves redox potential (NADPH) and energy transfer (\( \text{xyh, ptsG, fucO, phoB, deoR, dsbB} \) and \( \text{fda, encoding various carbohydrate transporter enzymes and oxidoreductases and their intermediates} \) was observed (Table 1). It is worth mentioning that \( \text{aceE, encoding a malate synthase}, \text{C1O30}_\text{RS02510, encoding an acetyltransferase (GNAT) family member, and kdpF, encoding a transport ATPase, were also activated by IHF (Table 1), suggesting that IHF may influence the tricarboxylic acid cycle and thus affect the growth of \( D. zeae \) MS2 (Figure S1).}

### 2.10 Deletion of IHF affects bacterial pathogenicity

The above characteristics, such as the Vfm QS system (Lv et al., 2019), the LuxR-solo system (Feng et al., 2019), CWDE (Hu et al., 2022), and the unknown phytotoxin (Hu et al., 2018), motility and biofilm formation, and the c-di-GMP level (Chen et al., 2016), are known to play important roles in \( Dickeya \) virulence. Thus, we hypothesized that IHF would mediate the virulence of \( D. zeae \) MS2. To determine whether deletion of \( \text{ihfA and ihfB} \) affected the invasive ability of MS2, we inoculated banana seedlings and potato slices with the same amount of MS2, \( \Delta \text{ihfA} \), \( \Delta \text{ihfB} \), \( \Delta \text{ihfAB} \) and the complemented strains. According to the virulence scoring method described previously (Feng et al., 2019), the disease severity of the banana seedlings inoculated with the mutants was ranked “0”
(symptomless) after 7 days of inoculation, while the banana seedlings inoculated with MS2 and the complemented strains were discoloured and rotten (Figure 6a). At 24 h postinoculation, the diseased area on the potato slices inoculated with the three deletion mutants was much smaller than those slices inoculated with MS2 or the complemented strains (Figure 6b), indicating that the deletion of ihf genes reduced the ability of MS2 to infect potato slices. We also measured the weights of the diseased tissue on potato slices caused by wild-type strain MS2 and its derivatives and calculated the colony-forming units (cfu) of the bacteria in the diseased tissues. The average weights of the diseased potato tissues inoculated by MS2, ΔihfA, ΔihfB, ΔihfAB, ΔihfA:ΔihfA, and ΔihfB:ΔihfB were 1.428, 0.049, 0.218, 0.014, 1.029, and 1.575 g, respectively. The cfu in each gram of diseased tissues was slightly less following inoculation with ΔihfA and ΔihfAB than those inoculated with the other strains (Figure S3). The result of the pathogenicity tests showed that deletion of ihf in D. zeae caused it to almost lose its pathogenicity to dicotyledonous and monocotyledonous hosts.

3 | DISCUSSION

In early studies, IHF was thought to be an integration protein in E. coli playing an important role in the integration of phage DNA into the host chromosome (Craig & Nash, 1984; Gamas et al., 1986; Yang & Nash, 1989). It can specifically recognize a consensus motif (5′-WATCAANNNTTR-3′) in the genome (Craig & Nash, 1984; Yang & Nash, 1989) and induce DNA bends. The conserved proline residues on the two subunits of IHF protein can be inserted between DNA base pairs, resulting in a sharp 140° distortion of DNA (Ali et al., 2001; Dixit et al., 2005; Ellenberger & Landy, 1997). By 2000, further functions of IHF had been discovered in bacteria. In Brucella abortus, IHF is involved in the transcriptional regulation of the virB operon of the T4SS (Seira et al., 2004). In Pseudomonas syringae pv. phaseolicola, IHF binds to the promoter of the phtD operon to mediate the synthesis of phaseolotoxin (Arvizu-Gómez et al., 2011). In Geobacter sulfurreducens, duplicated IhfA and IhfB proteins affect bacterial respiration, physiological function, and extracellular electron transfer (Andrade et al., 2021).

Although the gene coding sequences of IHF were described in D. dadantii 3937 in 1994 (Douillie et al., 1994), little research on the function of IHF was reported inDickeya until recently. In D. oryzae EC1, deletion of IHF resulted in decreased production of biofilms, CWDEs, and zeamines, and IHF was found to bind to the gene encoding a DGC, affecting the signal transduction of second messenger c-di-GMP (Chen et al., 2019). In D. dadantii 3937, IHF is capable of affecting the expression of genes required for virulence and pectin catabolism, and thus regulates the pathogenicity (Reverchon et al., 2021). The findings in this study showed a similar result in that IHF in D. zeae MS2 was required for virulence (Figure 6), production of CWDEs (Figure 3a), bacterial motility, and biofilm formation (Figure 4). Additional new functions of IHF were found, positively regulating the production of VFM and putrescine QS signals (Figures 1 and 2), indigoidine (Figure 1) and an MS2-specific phytotoxin (Figures 1 and 3b), and the secretion systems from T1SS to T6SS (Figure 1 and Table 1). IHF modulates diverse phenotypes in D. zeae MS2, for instance the biosynthesis of VFM QS signal (Figure 2b) by direct binding to the promoter of vfmE (Figures 1 and 7a), the putrescine signal level by direct interaction with the speA promoter and indirect interaction with spec (Table 1, Figures 1 and 7b), the luxR-solo system by direct interaction with the pipR promoter (Figure 7c), the production of CWDEs through multiple regulatory pathways, including IHF-VfmE, IHF-Fis/IHF-VfmE (Figure 7d), IHF-ArcAB-Fis (Figure 7e), IHF-SlyA (Figure 7f), and IHF-PrtD (T1SS)/IHF-GspC (T2SS) (Figure 7g), the production of indigoidine by direct binding to the indA promoter (Figure 7h), and the production of a phytotoxin by direct binding to the hdaA promoter (Figure 7i). Moreover, IHF directly bound the hrpL promoter and acted as an enhancer to activate hrpL expression, thus affecting T3SE secretion to the extracellular environment (Figure 7j), and regulated the expression of rhs, hecB, and hcp (Table 1 and Figure 7k). Cell motility and biofilm formation were also regulated by direct interaction of IhfA with the promoters of PilD (Figures 1 and 7l), the c-di-GMP encoding gene gcpJ, and the pilT (Figures 1, 5, and 7m). The findings in this study revealed many important target genes directly bound by IhfA protein, which encode pathogenicity regulatory factors manipulating and coordinating the virulence programming of D. zeae MS2, suggesting an important role of IHF as a master regulator in Dickeya. Interestingly, IhfA directly acted on the production of both VFM and putrescine QS signals to mediate CWDE production and flagella-triggered infection, respectively, which strongly contribute to the virulence of Dickeya (Lv et al., 2019; Shi et al., 2019).

In E. coli K12, IHF, along with Lrp, H-NS, Fis, FNR, CRP, and ArcA, are located in the top level of the transcriptional regulatory network from the regulonDB database (http://regulondb.cgg.unam.mx/menu/tools/transcriptional_regulation_network/images/NetWorkGene.jpeg) (Martínez-Antonio & Collado-Vides, 2003; Salgado et al., 2006). In this study, IHF has been found to interact with H-NS, Fis, and ArcA (Table 1, Figures 1 and 7). Such synergistic or antagonistic roles of IHF with other master regulators, even NAPs, have also been observed in other bacterial pathogens. In Salmonella enterica, IHF alleviates the inhibition of H-NS on hiA expression, which is important for pathogen virulence (Queiroz et al., 2011). Additionally, in E. coli, IHF assists EcPR to promote its binding to the ec promoter and activate fimbriae gene transcription. The interaction between IHF and EcPR can offset the inhibitory effect of H-NS on ec expression (Martínez-Santos et al., 2012). In S. marcescens FS14 and E. coli, ArcAB TCS regulates most of the genes in the tricarboxylic acid cycle and affects bacterial motility and virulence factor production (Loui et al., 2009; Zhang et al., 2018). This may also be another reason for the slow growth of ihf mutants in addition to the IHF-regulated genes involved in the metabolic process.

In Pseudomonas, PipR is a member of the LuxR-solo transcription factor family capable of responding to plant-produced compounds to mediate the expression of peptidase genes (Schafer et al., 2016). Although there is no evidence proving the direct reception of PipR to specific host elements, we believe that IHF is involved in the
plant–Dickeya transkingdom interactions through the LuxR-solo system. More associations need further in-depth research.

From the EMSA results, IhfA protein bound to DNA in the form of a monomer at low concentration and in the form of a polymer at high concentration in most cases (Figure 1). Some of the DNA regions that interacted with IhfA contain multiple putative IHF binding sites, such as the promoters of fis, hdaA, flhD, pilT, gcpJ, and arcB. However, there is no direct evidence that polymer formation of the IHF–DNA complex is specifically related to the number of binding sites. This nature of the IHF–DNA complexes is similar to those characterized in D. dadantii 3937 and Desulfovibrio vulgaris (Fiévet et al., 2014; Reverchon et al., 2021). IHF, as a NAP, binds DNA with loose specificity to modulate the transcriptional activity of bacterial genes by modification of the chromosome architecture (Reverchon et al., 2021). A recent study demonstrated that only the fully wrapped (147° of DNA bend) binding mode occurs with sequence specificity (Yoshua et al., 2021). It is expected that future research will reveal the mechanism of action of IHF and the discovery of more NAPs in microorganisms.

In summary, the findings from this study reveal the contribution of IHF to the pathogenic process, and dissect the regulation networks, in D. zeae MS2. IHF controls the production of VFM and putrescine QS signals by direct binding to the vfmE and speA promoters, affects phytotoxin and indigoidine production by direct binding to the hdaA and indA promoters, and interacts with the LuxR-solo system, Fis, SlyA, and FlhD transcriptional regulators directly, and with the T1SS–T6SS directly or indirectly. These findings highlight the importance of IHF in virulence regulation and add new insight to the sophisticated regulation pathways that control Dickeya virulence.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 | Bacterial growth conditions and antibiotic concentrations

The strains used in this study are listed in Table 2. D. zeae MS2 and its derivatives were cultured at 28°C in Luria-Bertani (LB) medium and minimal medium (MM) (Zhou et al., 2016). E. coli strains were cultured at 37°C in LB medium. Antibiotics were added to the medium at the following final concentrations when required: tetracycline (Tc) 15 μg/ml, streptomycin (Sm) 50 μg/ml, polymyxin B sulphate (Pm) 25 μg/ml, and kanamycin (Km) 100 μg/ml.

### Table 2 Strains and plasmids used in this study

| Strain or plasmid | Relevant description | Source or reference |
|-------------------|----------------------|---------------------|
| Strains
| Dickeya zeae |
| MS2 | Wild type of Dickeya zeae | Laboratory stock |
| ΔihfA | A deletion mutant derived from MS2 | This study |
| ΔihfB | A deletion mutant derived from MS2 | This study |
| ΔihfAB | A deletion mutant derived from MS2 | This study |
| ΔihfA::ihfA | ΔihfA containing plasmid construct pLAFR-ihfA, Tc<sup>a</sup> | This study |
| ΔihfB::ihfB | ΔihfB containing plasmid construct pLAFR-ihfB, Tc<sup>a</sup> | This study |
| Escherichia coli |
| CC118<sup>a</sup> | Δ(ara-leu) araDΔ lacX74 galE galK phoA20 thi-1 rpsE rpoE (Am) recA λpir | Laboratory stock |
| DH5<sup>a</sup> | supE44 ΔlacU169 (φ80lacZΔM15), hsdR17 recA1 endA1 grrA6 thi-1 relA1 λpir | Laboratory stock |
| BL21(DE3) | A protein expression host for efficient exogenous genes with T7 RNA polymerase as the expression system, Kan<sup>b</sup> | Laboratory stock |
| Plasmids |
| pKNG101 | R6K ori, sacB (Suc<sup>c</sup>), Str<sup>d</sup> | Laboratory stock |
| pET28b | N-His, N-thrombin, N-T7, C-His, Kan<sup>e</sup> | Laboratory stock |
| pRK2013 | Auxiliary vector, Kan<sup>e</sup> | Laboratory stock |
| pLAFR3 | Expression vector containing a tacTAC promoter, Tc<sup>c</sup> | Laboratory stock |

<sup>a</sup>Tc<sup>c</sup>, Kan<sup>e</sup>, Str<sup>d</sup> indicate resistance to tetracycline, kanamycin, or streptomycin, respectively. Suc<sup>c</sup> indicates sensitivity to sucrose.
4.2 Bacterial plasmids, mutant and complemented strain construction

Plasmids used in this study are listed in Table 2 and the primers used in this study are listed in Table S2. To knock out the ORF of *ihfA* and *ihfB*, 5' and 3' flanking fragments of *ihfA* and *ihfB* genes were amplified from MS2 genomic DNA. The two purified fragments and the BamHI/SpeI-digested suicide plasmid pKNG101 were ligated using a ClonExpress MultiS kit (Vazyme Biotech Co.). The ligation product was then transferred into the *E. coli* CC118Δ competent cells by 42°C heat shock and subsequently cultured in LB medium supplemented with 50 μg/ml Sm for approximately 4 h before spreading on LB agar supplemented with 50 μg/ml Sm. A positive transformant was co-cultured with wild-type MS2 and the helper plasmid pRK2013 by triparental mating (Lv et al., 2018). Mutants were selected on MM agar medium supplemented with 5% sucrose and confirmed by PCR and DNA sequencing. Similarly, triparental conjugation was used to delete *ihfB* based on the existing mutant ΔihfA to obtain the double-knockout mutant ΔihfAB. To construct the complemented strains, the coding regions of the genes *ihfA* and *ihfB* were amplified with the primers listed in Table S2 and then ligated to the BamHI/EcoRI-digested plasmid pLAFR3. The ligated DNA mixtures were separately transformed into *E. coli* DH5α competent cells, and positive transformants were then selected on LB agar supplemented with 15 μg/ml Tc. The recombinant plasmids were introduced into corresponding mutants by triparental mating. The complemented strains were selected on MM agar medium supplemented with 15 μg/ml Tc and confirmed by PCR. The deletion mutants and complemented strains were confirmed by DNA sequencing at Sangon Biotech.

4.3 Prediction of the IHF binding sites in the MS2 genome

fimo v. 5.1.0 was used to search for the IHF binding motif in the MS2 genome (GCF_002887555.1_ASM288755v1) (Grant et al., 2011). The motifs were filtered to identify those located at −600 to +100 bp from a CDS and harbouring ≥10 bp of A/T in the −26 to −14 bp upstream of the motifs.

4.4 RNA purification and RT-qPCR

MS2 and the mutants were cultured at 28°C in LB medium to an OD600 of 1.0. Total bacterial RNA was extracted using Eastep Super Total Extraction Kit (Promega), and the concentration of RNA was measured using a Nano-500 microspectrophotometer (Aosheng). Samples were visualized by agarose gel electrophoresis to confirm complete bands, and then stored at −80°C. Any contaminating genomic DNA was removed from the RNA samples using FastKing gDNA Dispelling RT SuperMix FastKing (Tiangen) and then cDNA was synthesized, and the resultant cDNA fragments were stored at −20°C. The qPCR was prepared using ChamQ SYBR qPCR Master Mix (Vazyme) according to the product instructions. The reaction procedure was performed in a QuantStudio 12K Flex real-time PCR instrument (Life Technologies). The qPCR primers used in the experiment are listed in Table S2 and the experimental data were processed using the 2−ΔΔCt method (Livak & Schmittgen, 2001).

4.5 EMSA

EMSA was performed using the methods in previous studies (Hu et al., 2022; Lv et al., 2019; Nasser & Reverchon, 2002). In detail, the coding region of *ihfA* was amplified with the primers listed in Table S2 using MS2 genomic DNA as the template. The amplified fragment was ligated to the BamHI/EcoRI-digested vector pET28b, transformed into *E. coli* BL21 (DE3) competent cells, and then grown on LB agar plates supplemented with 50 μg/ml Km overnight. The colonies were confirmed by PCR using the primers pET28b-F/R (Table S2). *E. coli* BL21 (DE3) containing the *ihfA*-coding fragment was grown at 37°C with 200 rpm shaking in 1 L of LB medium containing 50 μg/ml Km to OD600 of 0.6, then 1 mM IPTG was added into the medium and the cells were transferred to 18°C with shaking at 170 rpm to induce *ihfA* expression. All culture grown overnight was transferred into tubes for centrifugation at 1630 × g for 40 min and resuspended in appropriate volume of phosphate-buffered saline (PBS) twice. The cells were resuspended in 30 ml of PBS and disrupted by an ultrasonic cell breaker for 20 min with pulse for 6 s and pause for 2 s. The crude protein product after fragmentation by sonication was checked for the presence of the target protein on a 10% PAGE gel. Protein purification was performed at 4°C. The crude protein extract was mixed with Ni NTA beads. PBS containing low to high concentrations of imidazole (10, 50, 100, 150, 200, 300, and 500 mM) were added sequentially to the Ni NTA beads. The effluent at each concentration of imidazole was collected and analysed on a 10% PAGE gel. The protein samples were aliquoted into 1.5-ml tubes and stored at −80°C.

The promoter regions ranging from 200 to 300 bp of the target genes containing the IHF binding sites were amplified using the primers listed in Table S2. Purified promoter fragments were labelled using the biotin 3′-end labeling kit (Thermo Fisher). A reaction system with a total volume of 10 μl was then prepared using the LightShift Chemiluminescence EMSA kit (Thermo Fisher). PCR fluid contained 1 μl of LightShift 10X Binding Buffer, 0.5 μl of LightShift Poly (dI-dC), 1 nM labelled probe, 0–5 μM IhfA, and distilled water. The reaction mixture was incubated at 22°C for 30 min while the competition reaction mixture containing 100 nM unlabelled probe was incubated with the protein for 10 min before adding 1 nM labelled probe to complete the remaining reaction. The reaction product was run for 1–1.5 h in a 6% nondenaturing polyacrylamide gel at 90 V and then transferred onto a PVDF membrane at 80 V for 2.5 h. After cross-linking to the positively charged PVDF membrane by UV-light cross-linker (CX-200 UV Crosslinker), a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher) was used. The experimental results were observed with a chemiluminescence instrument (Tanon).
### 4.6 AHL and VFM bioassays

The AHL signal was detected using the method previously described (Hussain et al., 2008), where 20 ml of MM agar supplemented with 20 μl of X-Gal (40 mg/ml) was poured into a 9-cm diameter Petri dish under light-avoidance conditions. After solidification, the medium was cut into 1-cm wide separate slices. Agrobacterium tumefaciens CF11 containing a tra-lacZ fusion gene was used as a biosensor for the AHL signal. Equal amounts of freshly cultured CF11 at OD_{600} 0.1 were spotted on the separate slices. MS2, ΔihfA, and ΔihfB at OD_{600} 1.0 were spotted on the front of the CF11. The dish was incubated at 28°C for 24 h. The blue spots of the biosensor indicated the production of the AHL signal. To determine whether deletion of ihf genes affected VFM signal generation, the reporter strain with lacZ substituted for vfmE in ΔlacZ of D. oryzae EC1 was used in this study (Lv et al., 2019). First, 1 ml of MM was added to each well of a 24-well tissue culture plate (BIOFIL). Next, 1 μl of X-Gal and 10 μl of 6-h cultured reporter strain were added into each well. Then MS2 and ihf mutant supernatants in final concentrations of 5%, 10%, and 20% were added to the medium. The plate was shaken at 30°C and 200 rpm for 12 h. The assays were repeated three times in triplicate.

### 4.7 Measurement of PCWDE activity

The CWDE activity of the ihf mutants was measured as described previously (Chatterjee et al., 1995; Lv et al., 2018), but where the protease (Prt) assay medium was adjusted as a mixture of 3% agar LB medium and 4% skimmed milk. Thirty microlitres of cellulyase (Cel), pectate lyase (Pel), polygalacturonase (Peh), and protease (Prt) assay media were poured into each 13 × 13 cm square Petri dish. After drying in a vertical flow clean bench, 5-mm diameter holes were made on the solid media with a sterilized hole puncher. The agar in the holes was picked out with a sterilized toothpick. Fresh wild-type MS2, mutants, and complemented strains were grown to OD_{600} of 1.0 and the cultures were then centrifuged for 10 min at 13000 × g and 4°C. The obtained supernatants were filtered through a 0.22-μm membrane filter. Forty microlitres of the supernatants were added to the holes. The dishes were put in a 28°C incubator for 14–16 h. To observe the results, the Pel and Peh assay plates were soaked in 1 M HCl for 15 min, while the Cel assay plates were soaked in 0.1% (wt/vol) Congo red solution for 15 min. Subsequently, the three kinds of assay plates were washed twice with 1 M NaCl before observation. The Prt activity results were observed without any treatment. The radii of the halos were measured and collated with ImageJ v. 1.52a software. The assay was repeated three times in triplicate.

### 4.8 Phytotoxin assay

Previous studies revealed that D. zeae MS2 produces a novel phytotoxin as one of the virulence factors towards rice seeds and banana (Feng et al., 2019; Hu et al., 2018). To test whether IHF regulates this phytotoxin production, we first prepared a phytotoxin detection medium containing a mixture of 1.5% agar LB medium and 1% agarose. Next, 500 μl of fresh E. coli DH5α was mixed into 50 ml of phytotoxin detection medium (50–60°C) and poured into a 13 × 13 cm square Petri dish. Then 5-mm diameter holes were made on the solid medium with a sterilized hole puncher and 20 μl of supernatants of bacteria (OD_{600} of 1.0 in MM) were applied to the holes. Dishes were incubated overnight at 37°C. The radii of the clear bacterial inhibition zone on the medium were measured using ImageJ v. 1.52a. The assay was repeated three times in triplicate.

### 4.9 Measurement of cell motility and biofilm formation

Bacterial swimming and swarming motility were tested on semisolid medium according to the method described previously (Hussain et al., 2008). The medium recipe was slightly adjusted to accommodate the strain characteristics in this study. Specifically, 15 ml of swimming medium (Bacto peptone 5 g/L, NaCl 5 g/L, Bacto agar 2.5 g/L) was poured into a 9-cm diameter Petri dish. Strains were grown to OD_{600} 1.0 and 1 μl of bacterial cultures was stabbed into the centre of the semisolid medium after it was nearly air-dried. The same volume of bacterial cultures was added to the surface of the swimming medium (tryptone 10 g/L, NaCl 5 g/L, agarose 4 g/L) plates. The plates were put in a 28°C incubator for 10–12 h. The diameters of bacterial swimming and swarming were measured with ImageJ v. 1.52a. Twitching motility was tested based on the method described previously (Rashid & Kornberg, 2000). A bacterial colony from overnight 1.5% (wt/vol) LB agar plates was inoculated with a sterilized toothpick to the bottom of a fresh 1.5% (wt/vol) LB agar plate and cultured at 28°C for 24 h. The twitching area was measured by ImageJ v. 1.52a to calculate the diameter of the area.

To quantify the biofilms formed by MS2 and its mutants, strains were grown in LB medium to OD_{600} 1.0, 40 μl of which was added to 14 ml glass tubes containing 4 ml of SOBG medium (tryptone 20 g/L, yeast extract 5 g/L, MgSO_4 1.2 g/L, NaCl 0.5 g/L, KCl 0.186 g/L, glyc- erol 20 ml/L). The glass tubes were rested on a table for 48 h. Because biofilms produced by the wild-type MS2 and its derivatives do not stick well to the tube walls, we measured the nonadherent biofilms formed at the air/liquid interface. Under static conditions, biofilms of MS2 and its derivatives were formed on the surface of the SOBG medium. The thickness of the biofilms floating on the medium surface was measured using ImageJ v. 1.52a. The assay was repeated three times in triplicate. To quantitatively measure the attached biofilms, we modified the method described previously (O’Toole & Kolter, 1998). Briefly, cultures were removed and the glass tubes were washed by flowing water, then 200 ml of 0.1% crystal violet (wt/vol) was added into the glass tubes and incubated at room temperature for 15 min. The dye was removed and the glass tubes were washed by flowing water. The glass tubes were thoroughly dried at 60°C, then 1 ml of 95% ethanol was added to
analytically process, banana seedlings were first transplanted to plastic pots in an incubator at 28°C with 12-h alternating light–dark cycles. Fresh wild-type MS2, mutants, and complemented strains were cultured to OD<sub>600</sub> 1.0, then 200 μl of which was added to 1-ml sterile syringes, and injected into the base of the banana pseudostems. LB medium was used as the negative control. Treated banana seedlings were grown in an incubator at 28°C with 12-h alternating light–dark cycles for 14 days. Pictures were taken daily to record the disease incidence of the plants. Three biological replicates were set for each treatment.

The pathogenicity test on potato was performed as follows. First, healthy potatoes were washed, cut into slices about 5 mm thick, put into 9-cm diameter Petri dishes covered with clean filter paper, and then placed into a vertical-flow clean bench to air-dry the surface water. Next, the needle of a 1-ml sterile syringe was cut to approximately 1 mm in length and then stabbed into the centre of the potato slices to create a surface wound. Fresh wild-type MS2, mutants, and complemented strains were grown to OD<sub>600</sub> 1.0 and 2 μl of the culture was applied to the potato wound. LB medium was used as the negative control. Potato slices were incubated at 28°C for 24 h. The diseased area was measured using ImageJ v. 1.52a (Schneider et al., 2012). Each treatment was performed in triplicate.

To quantify the cfu of MS2 and its mutants in the potato slices, the diseased tissues were cut using a sterile knife, weighed and ground, and then diluted with sterile water, 100 μl of which was spread onto LB agar plates and cultured at 28°C for 24 h. Plates with colony numbers between 30 and 300 were selected to count the cfu. Each treatment was performed in triplicate.

**ACKNOWLEDGEMENTS**

This work was financially supported by grants from the National Natural Science Foundation of China (31972230), the Key-Area Research and Development Program of Guangdong Province (2020B0202090001 and 2018B020205003), the Natural Science Foundation of Guangdong Province, China (2020A1515011534), the Guangzhou Basic Research Program (202102080613), and the China Scholarship Council Fellowship Program Grant (202108440367).

**CONFLICT OF INTEREST**

The authors declare that there are no known conflicts of interest associated with this paper.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Chen, S., Hu, M., Hu, A., Xue, Y., Wang, S. & Liu, F. et al. (2022) The integration host factor regulates multiple virulence pathways in bacterial pathogen *Dickeya zeae* MS2. *Molecular Plant Pathology*, 23, 1487–1507.

Available from: [https://doi.org/10.1111/mpp.13244](https://doi.org/10.1111/mpp.13244)