Original Article

OhrR is a central transcriptional regulator of virulence in Dickeya zeae

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Abstract
Dickeya zeae is the causal agent of rice foot rot disease. The pathogen is known to rely on a range of virulence factors, including phytotoxin zeamines, extracellular enzymes, cell motility, and biofilm, which collectively contribute to the establishment of infections. Phytotoxin zeamines play a critical role in bacterial virulence; signaling pathways and regulatory mechanisms that govern bacterial virulence remain unclear. In this study, we identified a transcriptional regulator OhrR (organic hydroperoxide reductase regulator) that is involved in the regulation of zeamine production in D. zeae EC1. The OhrR null mutant was significantly attenuated in its virulence against rice seed, potato tubers and radish roots. Phenotype analysis showed that OhrR was also involved in the regulation of other virulence traits, including the production of extracellular cellulase, biofilm formation, and swimming/swarming motility. DNA electrophoretic mobility shift assay showed that OhrR directly regulates the transcription of key virulence genes and genes encoding bis-(3′–5′)-cyclic dimeric guanosine monophosphate synthetases. Furthermore, OhrR positively regulates the transcription of regulatory genes slyA and fis through binding to their promoter regions. Our findings identify a key regulator of the virulence of D. zeae and add new insights into the complex regulatory network that modulates the physiology and virulence of D. zeae.

Keywords
biofilm, c-di-GMP, Dickeya zeae, motility, pathogenicity, zeamines

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1 | INTRODUCTION

Dickeya spp. are important pathogenic bacteria that cause soft rot, wilts, and dwarfing diseases in a wide range of plants, including many important economic crops (Nasser et al., 2005). They secrete large amounts of plant cell wall-degrading enzymes (CWDEs), such as pectate lyases, cellulases, polygalacturonases, and proteases, via type I to III secretion systems (Hugouvieux-Cotte-Pattat et al., 2014; Kepseu et al., 2010; Reverchon et al., 2010; Yap et al., 2005). This process causes the destruction of plant tissues and gives rise to soft rot symptoms. Dickeya pathogens display a broad host range, which could be due to their ability to produce a wide range of virulence factors and also be associated with their genomic and genetic variations at genus and species levels (Zhou et al., 2015).

Dickeya zeae, previously named Erwinia chrysanthemi pv. zae, is the causal agent of maize stalk rot and rice foot rot diseases in many countries and regions (Hussain et al., 2008; Nassar et al., 1994; Samson et al., 2005; Sinha & Prasad, 1977). It is the only known member of the newly established Dickeya genus that can infect both monocotyledons and dicotyledons (Brady et al., 2012; Hussain et al., 2008; Nassar et al., 1996; Parkinson et al., 2014; Samson et al., 2005; Tian et al., 2016), whereas the others only infect dicotyledons or monocotyledons (Nassar et al., 1994; Sinha & Prasad, 1977).

It has been shown previously that D. zeae strain EC1 can strongly inhibit rice seed germination, even at a very low cell density, whereas other Dickeya pathogens such as D. dadantii strain 3937 did not display any inhibitory activity, even at a five-fold higher cell density than strain EC1 (Hussain et al., 2008). Subsequent studies have identified two phytotoxins, zeamine and zeamine II, that have strong inhibitory activities against rice seed germination and antimicrobial activities (Cheng et al., 2013; Liao et al., 2014; Wu et al., 2010; Zhou et al., 2011). The zeamine biosynthesis pathway is encoded by the zms gene cluster consisting of 18 coding genes, among which zmsA and zmsK have been characterized genetically and biochemically (Cheng et al., 2013; Zhou et al., 2011). Mutants of zmsA and zmsK are abolished in the production of zeamines and zeamine, respectively, and are significantly attenuated in virulence against rice seed germination (Cheng et al., 2013; Zhou et al., 2011). These findings suggest that zeamines are crucial for the virulence of D. zeae EC1, and therefore it is important to understand how the zms genes are regulated.

Previous studies identified several regulatory mechanisms of physiology and virulence in D. zeae. Deletion of the acyl-homoserine lactone (AHL) quorum-sensing system abolished AHL signal production and altered bacterial motility and biofilm formation, but had only a minimal effect on bacterial virulence (Hussain et al., 2008). Deletion mutants of the arginine decarboxylase gene speA, which encodes the synthase of the quorum-sensing signal putrescine, exhibit significantly attenuated bacterial motility, biofilm formation, and systemic infection in rice seeds (Shi et al., 2019). Deletion of the genes encoding two-component system (TCS) VfmHI, which is responsible for sensing and responding to the novel Vfm quorum-sensing signal, led to decreased production of zeamines and extracellular enzymes (e.g., CWDEs) in D. zeae EC1 (Lv et al., 2019). In addition to quorum-sensing systems, several transcriptional regulators involved in virulence regulation have also been unveiled. Deletion of slyA, which encodes a MarR family transcriptional regulator, resulted in markedly decreased zeamine production, biofilm formation, and pathogenicity on rice seeds (Zhou et al., 2016). Similarly, mutation of fis, which encodes a Fis family transcriptional regulator, caused a significant reduction in zeamine production, exoenzymes production, biofilm formation, cell motility, but significantly enhanced production of exopolysaccharides (EPS). Electrophoretic mobility shift assay (EMSA) results showed that Fis protein could directly bind to the promoters of zmsA and zmsK to regulate zeamine production (Lv et al., 2018). These findings provide a useful framework to further characterize and elucidate the regulatory networks governing the production of zeamines and other virulence factors in D. zeae.

To further understand the mechanisms modulating zeamine production, we screened a set of regulatory genes in D. zeae EC1 and identified another MarR family transcriptional regulator-encoding gene, ohrR, whose mutant showed altered patterns of zeamine production. Further functional characterization showed that OhrR can directly modulate the expression of genes involved in zeamine biosynthesis and also plays a crucial regulatory role in the production of extracellular cellulase and biofilm formation, as well as swimming and swarming motility. Furthermore, we showed that OhrR can positively regulate the expression of slyA and fis, and biosynthesis of the second messenger bis-(3’-5’) cyclic dimeric guanosine monophosphate (c-di-GMP) synthetases.

2 | RESULTS

2.1 | Deletion of ohrR in D. zeae EC1 reduces zeamine production

Zeamines are key virulence factors of D. zeae EC1 with potent antibacterial activity against a wide range of gram-negative and gram-positive bacterial pathogens (Cheng et al., 2013; Liao et al., 2015; Lv et al., 2018; Zhou et al., 2011). To identify additional regulators of zeamine production in D. zeae EC1, we screened the EC1 genome and found at least 185 genes encoding transcriptional factors and 74 genes encoding TCS proteins. We then generated single-gene deletion mutants for 10 selected genes encoding transcription factors or response regulator (RR) of TCSs (including MarR family transcriptional regulator, LuxR family transcriptional regulator, TetR family transcriptional regulator, and DNA-binding response regulator) and examined their zeamine production (Table S1). This led to the identification of the gene W909_17655, which encodes a MarR family transcriptional regulator that is 156 amino acids long and shares 72.06% protein sequence identity with the previously characterized OhrR in Xanthomonas campestris pv. campestris (RefSeq id WP_011035518.1). The deletion of W909_17655 resulted in significantly reduced zeamine production compared with the wild-type strain EC1 (Figure 1a,b) and deletion mutants of other regulatory genes examined in this study. In addition, the W909_17655 mutant and the wild-type strain...
EC1 showed similar growth curves in lysogeny broth (LB) and LS5 media (Figure S1). Transformation of the W909_17655 mutant with plasmids carrying the wild-type W909_17655 gene restored zeamine production to the level of wild-type strain EC1 (Figure 1a,b).

We noted that in X. campestris pv. campestris, OhrR is a transcriptional regulator of the expression of organic hydroperoxide reductase (Ohr), and thus was named as the organic hydroperoxide reductase regulator (OhrR) (Panmanee et al., 2002). To determine whether this function is conserved in D. zeae, we examined the sensitivity of the W909_17655 mutant to hydrogen peroxide. The assay results showed that the W909_17655 mutant became less sensitive to hydrogen peroxide toxicity than the wild-type strain EC1 when the hydrogen peroxide concentration was in the range of 0.5–1 mM (Figure 1c). The D. zeae gene W909_17655 was thus named as ohrR as well and further investigated for its roles in the regulation of zeamines and other virulence factors.

### 2.2 Deletion of ohrR results in decreased cellulase activity

Extracellular enzymes (e.g., cellulases, pectinases, and proteinases) are crucial for plant-pathogenic bacteria to dismantle structures of host cells and cause soft rot symptoms (Hugouvieux-Cotte-Pattat et al., 1996; Ma et al., 2007). Therefore, we analysed the production of extracellular enzymes using both qualitative and quantitative approaches. The results showed that the deletion of ohrR had no effect on pectinase and proteinase activities (Figure S2). In contrast, the cellulase activity in the ohrR mutant was reduced by about 4-fold compared with that of the wild-type strain and was restored by transformation with plasmids carrying the wild-type ohrR gene (Figure 2).

**FIGURE 1** The ohrR deletion mutant showed reduced antimicrobial activity compared to Dickeya zeae wild-type strain EC1. (a) Quantitative determination of zeamine production by strain EC1 and its derivatives. The concentration of zeamines was determined by this formula: zeamines (unit) = 0.5484e^{0.886x} (R^2 = 0.9957), where x is the radius in millimetres of the inhibition zone surrounding the well. For comparison, the data for the ohrR mutant and its complemented strain were normalized to that of the wild-type EC1, which was set to a value of 100%. (b) Qualitative detection of zeamine production strain EC1 and its derivative strains using plate assay. The antimicrobial activity bioassay plates were prepared as follows: 20 ml of 1% agarose containing 10^8 cells of Escherichia coli DH5α was overlayed onto the 120 × 120 mm plates containing 15 ml LB agar. The experiments were repeated three times in triplicate. (c) Hydrogen peroxide sensitivity assay. Strain EC1 and mutant ΔohrR were inoculated in LB medium containing different final concentrations of H_2O_2, as indicated, and measured by spectrophotometry at 600 nm with a microplate reader (BioTek). The experiments were repeated at least three times in triplicate. **p < 0.01. ***p < 0.001, ****p < 0.0001, Student’s t test

**FIGURE 2** The ohrR mutant decreased the production of extracellular cellulase. (a) Quantitative determination of the activity of extracellular cellulase of wild-type strain EC1 and its derivatives. (b) Cellulase activity detection on bioassay plates. The experiment was repeated three times and the error bars indicate the standard deviation. The final results of ohrR mutant were normalized to that of the wild-type EC1, which was set to a value of 100% for comparison. ***p < 0.001, Student’s t test

### 2.3 Deletion of ohrR enhances bacterial motility and decreases biofilm formation

Swimming and swarming are two different types of cell motility that aid bacteria in their territorial aggression and systemic infection.
Here, we investigated the role of *ohrR* in regulating the swimming and swarming motilities of *D. zeae* EC1. The results showed that the swimming and swarming motilities of the *ohrR* mutant were increased by about 1.2- and 0.9-fold, respectively, compared with those of the wild-type strain EC1 (Figure 3a,b). In trans expression of *ohrR* in the mutant restored the swimming and swarming motilities to the wild-type level (Figure 3a,b).

To investigate the function of OhrR in modulating biofilm (attached bacterial cells) formation, we quantified adhered biofilm biomass using crystal violet staining. The results showed that the biofilm formation of *ohrR* mutant was substantially reduced by about 3-fold compared with the wild-type EC1 and complemented strains (Figure 3c).

Our previous study showed that the second messenger c-di-GMP has a key role in the modulation of bacterial motility and biofilm formation. In addition, several *D. zeae* genes encoding key c-di-GMP synthetase (*W909_14945*) or degrading enzymes (*W909_14950* and *W909_10355*) have been shown to be involved in the regulation of cell motility (Chen et al., 2016). Therefore, we examined the concentration of intracellular c-di-GMP in the wild-type EC1, the *ohrR* deletion mutant Δ*ohrR*, the complemented strain Δ*ohrR* (ohrR), the *ohrR* mutant transformed with *W909_14945* (Δ*ohrR* [14945]), and the *W909_14945* deletion mutant Δ14945. The results showed that, compared with the wild-type strain EC1, deletion of *ohrR* and *W909_14945* led to about 27- and 25-fold reduction in the intracellular concentration of c-di-GMP, respectively (Figure 3d). Additionally, the intracellular c-di-GMP concentration of Δ*ohrR* was partially recovered when complemented with *ohrR* and became 8.97-fold higher than that of the wild-type EC1 when transformed with *W909_14945* (Figure 3d). Notably, the swarming and swimming motilities, as well as the biofilm formation capacity of Δ*ohrR* (14945), were comparable to those of the wild-type strain (Figure 3a–c). Altogether, these results indicate that OhrR plays an important role in regulating the intracellular

![Figure 3](image-url)
homeostasis of c-di-GMP in D. zeae, which in turn affects motility and biofilm formation.

2.4 | Deletion of ohrR decreases the virulence of D. zeae EC1

D. zeae strain EC1 can infect both monocotyledons and dicotyledons, and inhibit rice seed germination at a low cell density (Hussain et al., 2008; Sinha & Prasad, 1977). To test the virulence of ohrR mutant, we treated rice seeds with 10 ml of sterile water containing bacterial cells at $10^3$ cfu. Rice seed germination rate was determined 1 week after inoculation. The ohrR mutant did not show a significant inhibitory effect against rice seed germination (Figure 4a,b); the lengths of shoots and roots germinated from treated seeds were similar to that of the water-treated control (Figure 4c). In contrast, treatment with either the wild-type EC1 or the complemented strain ΔohrR(ohrR) resulted in total inhibition of rice seed germination (Figure 4a,b).

Because D. zeae EC1 can also infect dicotyledons, we examined the virulence of wild-type EC1, ΔohrR, and ΔohrR(ohrR) on potato and radish by inoculating 1 µl of bacterial cells (OD$_{600}$ = 1.0) on corresponding tuber/root slices. The results showed that the deletion of ohrR significantly reduced the maceration zones by about 67% and 83% compared to the wild-type strain EC1 and the complemented strain (Figure 4d,e).

2.5 | Deletion of ohrR alters the expression levels of key virulence genes

The gene zmsK is essential for the biosynthesis of zeamine II (Cheng et al., 2013), and the deletion of zmsA abolished the production of both zeamine and zeamine II by D. zeae EC1 (Zhou et al., 2011). Our reverse transcription quantitative PCR (RT-qPCR) analysis showed that the expression levels of zmsA and zmsK were significantly reduced by 2.9- and 2.7-fold, respectively, in the ohrR mutant compared with the wild-type strain EC1. Similarly, we also found that the expression levels of zmsC and zmsD, two other genes in the zms cluster that encode a hypothetical protein and a 3-oxoacyl-ACP reductase (Cheng et al., 2013; Zhou et al., 2015), respectively, were markedly decreased by 2.48- and 3.62-fold compared with the wild-type EC1 (Figure S3). Consistent with the reduced expression of zms genes, zeamine production was decreased by about 95% and 94% in the deficiency mutants of zmsC and zmsD, respectively (Figure S4).

In addition to the genes associated with zeamine biosynthesis, we also compared the expression levels of various other virulence genes in the wild-type strain and the ohrR mutant, including celZ, which encodes an endoglucanase (Zhou et al., 2015), bssS, which is involved in biofilm formation (Zhou et al., 2015) (Figure S5), W909_14945, which encodes a c-di-GMP synthetase (Zhou et al., 2015), and W909_14950 and W909_10355, which encode...
two c-di-GMP degrading enzymes (Chen et al., 2016). The results showed that, in the ohrR mutant, the expression levels of celZ, bssS, and W909_14945 were significantly reduced by 4.26-, 1.86-, and 1.73-fold, respectively (Figure S3), whereas the expression levels of W909_14950 and W909_10355 were increased by 1.25- and 1.52-fold, respectively (Figure S3).

2.6 | OhrR directly interacts with the promoters of a range of virulence genes

To further understand the regulatory mechanisms of OhrR, we carried out EMSA using purified OhrR protein and amplified genomic fragments corresponding to the promoter regions of putative target genes of OhrR. The results showed that OhrR could interact with the promoters of zmsA, celZ, bssS, W909_14945, and W909_10355, indicating that OhrR could directly modulate the transcriptional expression of these virulence genes and thus regulate the zeamine biosynthesis, CWDEs production, biofilm formation, and c-di-GMP metabolism (Figure 5). As a negative control, OhrR could not bind to the promoter of W909_04705, which encodes a serine protease (Figure 5 and Figure S3).

2.7 | OhrR modulates the expression of key virulence regulators SlyA and Fis

To dissect the regulatory networks involving OhrR, SlyA, and Fis, we generated single (ΔohrR, Δsly, and Δfis), double (ΔohrRslyA and ΔohrRfis), and triple (ΔohrRslyAfis) mutants of the genes through deletion mutagenesis and measured their zeamine production. The results showed that (Figure 6b and Figure S1b) (a) all three single-deletion mutants showed comparable reductions (66%–70%) in their zeamine production; (b) compared to single-deletion mutants, the zeamine production of ΔohrRslyA was decreased by about 40% (vs. ΔohrR) and 45% (vs. ΔslyA), while that of the ΔohrRfis was decreased by about 51% (vs. ΔohrR) and 35% (vs. Δfis); and (c) compared to double-deletion mutants, the zeamine production of ΔohrRslyAfis was reduced by about 47% (vs. ΔohrRslyA) and 34% (vs. ΔohrRfis).

The growth dynamics analysis showed that all single, double, and triple mutants as well as the wild-type strain EC1 exhibited similar growth dynamics in both LS5 and LB media (Figure S1). These findings suggest a cumulative effect of these transcriptional regulators in their modulation of zeamine production.

To further understand the regulatory relationship among ohrR, slyA, and fis, their expression levels in each of the slyA, fis, and ohrR mutants were measured. The results showed that (Figure 6b and Figure S1b) (a) all three single-deletion mutants showed comparable reductions (66%–70%) in their zeamine production; (b) compared to single-deletion mutants, the zeamine production of ΔohrRslyA was decreased by about 40% (vs. ΔohrR) and 45% (vs. ΔslyA), while that of the ΔohrRfis was decreased by about 51% (vs. ΔohrR) and 35% (vs. Δfis); and (c) compared to double-deletion mutants, the zeamine production of ΔohrRslyAfis was reduced by about 47% (vs. ΔohrRslyA) and 34% (vs. ΔohrRfis).
single-gene deletion mutants were determined by RT-qPCR. The results showed that the expressions of slyA and fis were decreased by about 6.3- and 2.7-fold, respectively, in the ohrR mutant, and the expression of fis was decreased by 4.0-fold in the slyA mutant (Figure 6a). In contrast, deletion of slyA and fis did not seem to have a significant impact on the expression of ohrR (Figure 6a) and, similarly, deletion of fis had no effect on slyA expression (Figure 6a).

Consistent with the above findings, the expressions of the zeamine synthesis genes zmsA and zmsK were lower in the double and triple deletion mutants than in the corresponding single mutants (Figure 5d). In trans expression of fis and slyA, respectively, in the ohrR mutant rescued zeamine production, biofilm formation, and swimming motility to wild-type levels (Figure 6c, Figures S1a and S7a,b). We also found that expression of fis in the slyA mutant restored the production of zeamines to the level of wild-type strain EC1 (Figure 6c).

3 | DISCUSSION

Previous studies have unveiled that OhrR is a key regulator for sensing and responding to oxidative stress in various bacterial species (Atichartpongkul et al., 2016; Chandrangsu et al., 2018; Gaballa et al., 2014; Garnica et al., 2017; Liu et al., 2016). Recently, OhrR was also shown to influence other phenotypes, such as the production of c-di-GMP in Chromobacterium violaceum (Previato-Mello et al., 2017) and the biosynthesis of avermectin in Streptomyces avermitiliswe (Sun et al., 2018). In this study, we showed that OhrR of D. zeae not only has a conserved function in the response to oxidative stress (Figure 1c), but also plays a key role in the regulation of virulence traits. These findings indicate that D. zeae EC1 heavily relies on OhrR in the modulation of its physiology and pathogenicity.

In the modulation of response to oxidative stress, OhrR is a transcriptional repressor, which acts by directly binding to the promoters and represses transcription of the genes encoding peroxidase genes. Exposure to hydrogen peroxide, which is a common type of oxidative stress at the early stage of pathogenic infection, leads to oxidation and structural changes of OhrR that disrupt its binding to promoters and thus resume the expression of the target genes (Atichartpongkul et al., 2010, 2016). This mode of regulation seems to be well-conserved in D. zeae EC1, as the deletion of ohrR resulted in enhanced bacterial survival in the presence of a high dosage of H$_2$O$_2$ (Figure 1c). At the same time, OhrR could also function in a positive way in modulating certain virulence traits, such as zeamine production.
and cellulase production. Our results showed that the purified OhrR could directly bind to the promoters of the genes involved in zeamine biosynthesis and cellulase production (Figure 5), and that deletion of ohrR resulted in markedly reduced production of zeamines and cellulase (Figures 1a and 2a). Given that an oxidative environment is a common stress that bacterial pathogens might encounter, especially at the initial stage of infection, it remains unclear whether and how oxidative stress would affect the OhrR-mediated regulation of zeamine and cellulase production, which is worthy of further investigation.

The ubiquitous second messenger c-di-GMP is involved in modulating a range of important biological functions and behavior in many bacterial pathogens, including biofilm formation, cell motility, and morphology (Boehm et al., 2010; Chen et al., 2016; Hengge, 2009; Ryjenkov et al., 2006). Understanding the regulatory mechanisms of c-di-GMP can not only help to predict the behavior of pathogens but also aid in developing effective therapeutic strategies to prevent bacterial infections (Abdul-Sater et al., 2013; Valentini & Filloux, 2019). The intracellular level of c-di-GMP in bacterial cells is governed by both diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which contain GGDEF and EAL (or HD-GYP) catalytic domains for synthesis and degradation of c-di-GMP, respectively (Boehm et al., 2010; He et al., 2007; Ryjenkov et al., 2006; Ryan et al., 2006). In Dickeya species, several key c-di-GMP metabolic enzymes in modulating motility, biofilm formation, and CWDEs production have been characterized, including two PDEs (EcPB and EcPC) in D. dadantii 3937 (Yi et al., 2010) and one DCG (encoded by W909_14945) as well as two PDEs (encoded by W909_10355 and W909_14950) in D. zeae EC1 (Chen et al., 2016, 2020). However, the regulation of these c-di-GMP metabolic enzymes has not yet been fully understood.

The results of this study provide a new insight in this regard. We showed that deletion of ohrR led to a dramatically lower concentration of c-di-GMP, which is accompanied by significantly decreased expression of DGC-encoding gene W909_14945 and markedly enhanced expression of PDE-encoding genes W909_10355 and W909_14950 (Figure S3). In addition, the in trans expression of W909_14945 in the ohrR mutant restored biofilm formation and cell motility to a level comparable with that of the wild type (Figure 3a–c). These findings strongly suggest that OhrR influences the homeostasis of intracellular c-di-GMP and, in turn, downstream traits such as biofilm formation and cell motility mainly through regulating the expression of c-di-GMP metabolic genes. Furthermore, the regulation is at least partially direct, as our EMSA assays showed that OhrR could directly interact with the promoter regions of W909_10355 and W909_14945 (Figure 5). Interestingly, different from the repressive role of OhrR on c-di-GMP synthase in C. violaceum (Previanto-Mello et al., 2017), deletion of ohrR dramatically decreased the intracellular c-di-GMP levels in D. zeae EC1 (Figure 3d).

Similar to OhrR, SlyA and Fis are both global regulators of largely the same set of virulence traits in D. zeae, including biofilm formation, bacteria motility, and the production of zeamine, CWDEs, and EPS (Lv et al., 2018; Zhou et al., 2016). Therefore, we conducted systematic genetic analyses to dissect the relationships among the three regulators. The double mutants ΔohrRslyA and ΔohrRfis showed decreased zeamine production compared to the single mutants, while the triple mutant ΔohrRslyAΔfis was the lowest among all mutants (Figure 5c), which is consistent with the expression patterns of zms genes in these mutants (Figure S6). These findings suggest a cumulative effect of OhrR, SlyA, and Fis in the regulation of zeamine production. Importantly, the results of our RT-qPCR and EMSA assays suggest that OhrR is upstream in the regulatory network and can directly modulate the expression of slyA and fis (Figures 5 and 6a–c). In line with this, in trans expression of either fis or slyA in ohrR mutant could rescue its zeamine production, biofilm formation, and swimming motility to wild-type levels (Figure 6c and Figure S7a,b). Similarly, in trans expression of fis in slyA mutant can restore its zeamine production to the level of wild-type strain EC1 (Figure 6c).

Altogether, our results indicate that OhrR, SlyA, and Fis constitute a complex regulatory network of zeamine production, bacterial motility, and biofilm formation, in which OhrR plays a central regulatory role (Figure 7), and this regulation mechanism provides a reference for the study of other pathogenic bacteria.

The roles of OhrR in sensing and responding to oxidative stress are widely conserved in many bacteria (Agrobacterium tumefaciens, Azorhizobium caulinodans, Bacillus subtilis, D. dadantii, Mycobacterium smegmatis, Pseudomonas aeruginosa, S. avermitilis, X. campestris) (Atichartkongkul et al., 2016; Chandrangu et al., 2018; Chuchue et al., 2006; Gaballa et al., 2014; Garnica et al., 2017; Grenier et al., 2006; Liu et al., 2016; Panmanee et al., 2002; Reverchon et al., 2010; Si et al., 2020). At the same time, OhrR has been found to be a key regulator of virulence in some pathogenic bacteria (Atichartkongkul et al., 2010; Das et al., 2019; Pande et al., 2018; Previanto-Mello et al., 2017). SlyA, another member of the MarR family, is also a global regulator of antibiotic resistance and multiple virulence traits, including type III secretion system (T3SS), swimming motility, pellicle formation, the production of CWDEs, EPS, and zeamines, and the ability to cause disease to the host (Alexshun et al., 2000; Haque et al., 2009; Wilkinson & Grove, 2006; Zhou et al., 2016; Zou et al., 2012). Similar to SlyA, the transcriptional regulator Fis also plays a crucial role in regulating the production of various virulence factors (e.g., the CWDEs, zeamines, EPS, cell motility, pellicle-biofilm formation, and cell aggregation) in a variety of pathogenic bacteria (Falconi et al., 2001; Goldberg et al., 2001; Lautier & Nasser, 2007; Lenz & Bassler, 2007; Lv et al., 2018; Ó Cróinín et al., 2006; Ouafa et al., 2012; Prigent-Combaret et al., 2012; Schechter et al., 2003). However, although OhrR, SlyA, and Fis are all important regulators of virulence, the regulatory relationships among them were not investigated.

Besides OhrR, SlyA, and Fis, several other regulators have also been reported in Dickeya. For instance, MfbR is a newly identified MarR family transcriptional regulator that activates genes encoding CWDEs in D. dadantii 3937 (Reverchon et al., 2010). Other master regulators, including PecS, PecT, KdgR, H-NS, the GacAS, and PhoPQ two-component systems, and the quorum-sensing system Vfm, are also known to be associated with the regulation of pel genes and the production of CWDEs (Reverchon & Nasser, 2013; Reverchon et al., 2010; Grenier et al., 2006; Yang et al., 2008). However, the
roles of these regulators have not been examined yet in *D. zeae* EC1. Therefore, it is worth investigating the relationships among these regulators and OhrR, SlyA, and Fis in *D. zeae* EC1, and further elaborating the virulence regulatory network we report in this study.

In summary, the results of this study demonstrate that OhrR is a global regulator that is critical to a wide range of virulence traits in *D. zeae* EC1, including zeamine and cellulase production, cell motility, biofilm formation, and virulence on monocotyledons and dicotyledons. In particular, we showed that OhrR has both positive and negative regulatory roles on gene expression in *D. zeae*. Furthermore, we present evidence that OhrR and two other global regulators, SlyA and Fis, constitute a transcriptional regulatory network in which OhrR is positioned upstream of SlyA and Fis and can positively regulate their expression. These findings would help us to better understand the complex regulatory mechanisms that control the physiology and virulence of *D. zeae* and related phytopathogens.

### 4 EXPERIMENTAL PROCEDURES

#### 4.1 Bacterial strains and growth conditions

The plasmids, wild-type strain *D. zeae* EC1, and its derivatives used in this study are listed in Table 1. *Escherichia coli* strains DH5α (TransGen Biotech) and CC118β were used as a host in gene cloning and vector construction and were grown at 37°C in LB medium. *D. zeae* strain EC1 and its derivatives were cultivated at 28°C in LB medium, minimal medium broth (MM) (Cheng et al., 2013), or LS5 medium as indicated (Liao et al., 2014). Antibiotics were added to the medium at the following final concentrations when required: polymyxin B sulphate (pB) 25 µg/ml, streptomycin (Str) 50 µg/ml, kanamycin (Kan) 50 µg/ml, ampicillin (Amp) 100 µg/ml, and tetracycline (Tc) 15 µg/ml.

#### 4.2 Deletion mutagenesis and complementation

Deletion mutants of target genes were generated using the primers listed in Table S2 to amplify the DNA fragments flanking its coding sequences, with primers 1 and 2, 3 and 4, respectively. The two fragments were then fused using primers 1 and 4. The PCRs in this study were performed using the high fidelity Taq DNA polymerase (Phanta Super Fidelity DNA Polymerase). The fusion fragment and the suicide plasmid pKNG101 were digested, respectively, with restriction enzymes *Spe*I and *Bam*HI, purified by using Cycle Pure Kit (Omega), and then ligated together by using T4 DNA ligase (NEB). The products of ligation were transformed into *E. coli* CC118β competent cells by heat shock at 42°C and the bacterial cells were cultured at 37°C for 4 hr. The transformants were selected on LB solid medium plate supplemented with Str and verified using the primers pKNG-F/pKNG-R by PCR analysis. The recombinant plasmids were introduced into *D. zeae* EC1 by using triparental mating as described previously to generate in-frame deletion mutants (Lv et al., 2018). The mutants were selected on the MM solid medium plate containing 5% sucrose and pB, and conformed by PCR and DNA sequencing.

To construct vectors for the complementation of deletion mutation strains, the DNA fragment containing the promoter sequence (803 bp) and coding sequence of *ohrR* was amplified using the primers *ohrR*-BF/*ohrR*-HR. The purified PCR products and the expression
vector pBBRlMCS4 were digested with restriction enzymes BamHI and HindIII, and purified again prior to ligation and transformed into E. coli DH5α competent cells (TransGen Biotech). The transformants were selected on LB solid medium plate containing Amp and confirmed by PCR using primers MCS4-F and MCS-R, and DNA sequencing. Triparental mating was performed to introduce the recombinant plasmid into ohrR mutant as described above. The complemented strains were selected on MM solid medium plates containing Amp and pB, and confirmed by PCR analysis.

4.3 | Zeamine production bioassay

The antimicrobial bioassay plates were prepared by pouring 15 ml of LB agar medium onto a 120 × 120 mm plate, and then overlayed with 1% agarose 20 ml containing 10⁸ cells of E. coli DH5α. Wells of 5 mm diameter were punched into the bioassay plates after solidification. D. zeae bacterial culture was grown in LS5 medium (Liao et al., 2014) at 28°C to OD₆₀₀ around 1.4, which was centrifuged twice at 13,500 × g for 10 min, and 20 µl of supernatants was added into the wells. The assay plates were incubated at 37°C for 10 h. The antimicrobial activity was determined by measuring the radius of the visible clear zone surrounding the well. The concentration of zeamines was determined by the formula zeamines (unit) = 0.5484e⁰.⁸₈₆₆x, the correlation coefficient (R²) is 0.9957, and x is the width in millimetres of the inhibition zone surrounded the well (Chen et al., 2016; Cheng et al., 2013; Liao et al., 2014; Lv et al., 2018; Zhou et al., 2011).

4.4 | The hydrogen peroxide tolerance assay

Aliquots of bacterial cultures (OD₆₀₀ = 1.50 ± 0.05, 1.5 µl) were inoculated into each well of a 96-well microtitre plate containing 150 µl of fresh LB medium that contained hydrogen peroxide (H₂O₂) at a final concentration of 0.1-2 mM as indicated, with four replicates per treatment and repeated twice. The plate was incubated at 28°C with shaking at 200 rpm for 16 h. The optical density at 600 nm (OD₆₀₀) of the bacterial culture was measured by a microplate reader (BioTek).

TABLE 1 | Strains and plasmids used in this study

| Strains or plasmids | Relevant phenotypes and characteristics | Source or reference |
|---------------------|----------------------------------------|-------------------|
| Strains             |                                        |                   |
| EC1                 | Wild type of Dickeya zeae, pB’         | Laboratory collection |
| ΔohrR               | A deletion mutant derived from EC1, pB’ | This research     |
| ΔohrR(ohrR)         | ΔohrR containing ohrR coding region at the downstream of lacZ promoter, Amp’, pB’ | This research     |
| ΔslyA               | A deletion mutant derived from EC1, pB’ | This research     |
| Δfis                | A deletion mutant derived from EC1, pB’ | This research     |
| ΔohrRfis            | A double-deletion mutant derived from EC1, pB’ | This research     |
| ΔohrRsilA           | A double-deletion mutant derived from EC1, pB’ | This research     |
| ΔohrRsilAslyA       | A triple-deletion mutant derived from EC1, pB’ | This research     |
| ΔohrR(14945)        | ΔohrR containing pBBRI-14945 recombinant vector, Amp’, pB’ | This research     |
| Δ14945              | A deletion mutant derived from EC1, pB’ | Laboratory collection |
| ΔzmsC               | A deletion mutant derived from EC1, pB’ | Laboratory collection |
| ΔzmsD               | A deletion mutant derived from EC1, pB’ | Laboratory collection |
| ΔbssS               | A deletion mutant derived from EC1, pB’ | This research     |
| CC18β               | Escherichia coli strain as host for plasmid constructs derived from pKNG101 | Laboratory collection |
| DH5α                | E. coli strain as host for plasmid constructs derived from pBBR1-MCS4 | Laboratory collection |
| HB101[pRK2013]      | Thr leu thi recA hsdR hsdM pro, Kan’ | Laboratory collection |

| Plasmids            | Relevant phenotypes and characteristics | Source or reference |
|---------------------|----------------------------------------|-------------------|
| pKNG101             | Knockout vector, Str’                 | Laboratory collection |
| pKNG101-ohrR        | pKNG101 carries the in-frame deleted fragment of ohrR, Str’ | This research     |
| pKNG101-slyA        | pKNG101 carries the in-frame deleted fragment of slyA, Str’ | This research     |
| pKNG101-fis         | pKNG101 carries the in-frame deleted fragment of fis, Str’ | This research     |
| pGEX-6p-ohR         | pGEX-6p-1 carries the ohrR coding region, Amp’ | This research     |
| pBBR1-MCS4          | Expression vector contains a lacZ promoter, Amp’ | This research     |
| pBBR1-14945         | pBBR1-MCS4 carries the coding region of 14945 downstream of lacZ promoter, Amp’ | Laboratory collection |
| pBBR1-ohrR          | pBBR1-MCS4 carries the coding region of ohrR downstream of lacZ promoter, Amp’ | This research     |

*pB’, Amp’, Kan’, Str’ , Tc’ = resistance to polymyxin B sulphate, ampicillin, kanamycin, streptomycin, or tetracycline, respectively.
4.5 | Cellulase activity assay

The cellulase activities of the culture supernatants of *D. zeae* EC1 and its derivatives were determined by using the enzyme activity detection plate, following the methods described previously (Caldas et al., 2002; Chatterjee et al., 1995; Lv et al., 2018). The cellulase bioassay plates were prepared by pouring 35 ml of medium (containing 1 g carboxymethyl cellulose sodium, 3.8 g Na₂PO₄, and 8 g agarose, pH 7.5) into 120 x 120 mm plates, and wells of 5 mm were punched after solidification. Then 20 μl of supernatants of bacteria culture, which were grown to OD₆₀₀ = 1.3 and centrifuged at 13,500 × g for 10 min, were added to the wells. Plates were incubated at 28°C for 14 h and then the bioassay plates were stained with 0.1% Congo red (wt/vol) and decoloured with 1 M NaCl. Quantitative determination was performed as described above. The supernatant of 1 ml of bacterial cultures was added to a glass containing 3 ml of 0.5% carboxymethyl cellulose sodium solution and mixed uniformly. The tubes were incubated in a 50°C water bath for 30 min. A solution of 3,5-dinitrosalicylic acid was added to the tubes and boiled for 10 min, and then diluted 5-fold and quantified by the absorbance at 550 nm after the reaction mixture had cooled. A tube without bacterial culture supernatant was used as a negative control. Three independent assays were carried out for each bacterial strain (Lv et al., 2018).

4.6 | Biofilm formation assay

Biofilm formation assays were performed as described previously (Burova et al., 1983; Deng et al., 2011; Dong et al., 2008; Lv et al., 2018). Overnight bacterial cultures were diluted in SOBG (super optimal broth plus glycerol) medium to OD₆₀₀ = 0.01 and 100 μl of diluted cultures was added to each well of a 96-well microtitre plate. The plate was incubated at 28°C with shaking at 150 rpm for 18 h. The cultures were emptied and 150 μl of 0.1% crystal violet (wt/vol) solution was added to each well. The plate was kept at room temperature for 20 min before the dye was removed and each well was washed three times using distilled water. The remaining crystal violet was dissolved by adding 200 μl of 95% ethanol, and quantification of the attached bacterial cells (biofilm) was performed by measuring spectrophotometric values at 595 nm with a microplate reader (BioTek).

Collective swimming motility was assessed in a semisolid medium (containing 10 g Bacto tryptone, 5 g NaCl, and 2 g agar per litre) plate. A bacterial culture grown overnight (1 μl) was spotted on the centre of the plates and incubated at 28°C for 18 h before measurement. Collective swarming motility was detected as previously described (Lv et al., 2019). Each experiment was repeated at least three times in triplicate.

4.7 | RNA purification and RT-qPCR analysis

Cultures of *D. zeae* EC1 and ohrR mutant in LB and LS5 media (OD₆₀₀ = 1.0) were centrifuged and the total RNA was isolated by the SV total RNA isolated system kit (Promega). Genomic DNA was removed by treating with DNase I (Takara) at 37°C for 1 h and was confirmed by PCR using the 16S primer pair and purified using the RNA clean kit (Qiagen). The concentration of RNA was measured using a NanoDrop ND-100 spectrophotometer and the integrity of RNA was visualized by agarose gel electrophoresis.

For RT-qPCR analysis, SuperReal PreMix Color SYBR Green, 2× (Tiangen Biotech Co. Ltd) was used on QuantStudio 6 Flex (Applied Biosystems) following the user’s guide from the manufacturer. The high-quality primer amplification capability was determined by melting curve analysis. The absolute value of −ΔΔCt = (ΔCt₁ − ΔCt₂) was calculated as described in the formula 2 −ΔΔCt (Livak & Schmittgen, 2001). The RT-qPCR experiment was repeated at least twice and the cDNA samples were prepared from triplicate cultures each time.

4.8 | Protein–DNA interaction assay

For OhrR purification, the coding region of ohrR was amplified by PCR using primers pGEX-6p-ohrR-F and pGEX-6p-ohrR-R (Table S1), and the vector was linearized by BamHI and EcoRI. The resultant 510 bp DNA fragment of the ohrR coding region was cloned into the BamHI-EcoRI region of vector pGEX-6p-1 by ClonExpress MultiS (Vazyme) to generate pGEX-6p-ohrR (Table S2). The OhrR-GST protein was induced and purified following the method described previously (Lv et al., 2018). The *E. coli* BL21 cells containing pGEX-6p-ohrR were induced to express OhrR by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM at 18°C overnight. The bacterial cells were collected and disrupted using Stansted Fluid Power at 120 psi and the crude protein extracts were prepared by centrifugation at 15,000 × g for 20 min. Protein purification was performed following the protocol described in the Glutathione Resin User Manual (Clontech) and the protein samples were stored at −80°C.

The DNA sequences of target promoter regions were amplified by PCR using the primers listed in Table S1. The purified PCR products were labelled by biotin using a Biotin 3′ End DNA Labeling Kit (Thermo). The reaction mixture contained 1 μM or 2 μM OhrR protein as indicated, and 20 fmol labelled oligonucleotide fragments in a final volume of 10 μl. The protein–DNA complexes and the unbound free DNA fragments were separated on a 6% nondenaturing polyacrylamide (acrylamide/bisacrylamide 29:1 vol/vol) gel using Tris-borate-EDTA (TBE) electrophoresis buffer and were detected using chemiluminescence (Tanon). The specific interaction of OhrR protein–DNA fragments was verified by incubation of 100- and 150-fold molar excess of unlabelled DNA fragments with OhrR protein before the addition of labelled DNA fragments. The purified glutathione S-transferase (GST) protein that could not bind to the promoter sequences was used as a blank control (Lv et al., 2018).

4.9 | Quantitative analysis of c-di-GMP

Cells were grown overnight in LB medium and adjusted to OD₆₀₀ = 2.0, then subcultured to 4 ml of MM with 50 times dilution...
in a 14-mL culture tube (Crystalgen). When the bacterial culture reached an OD$_{600}$ of 0.4–0.6, 1 mL samples were transferred to a 2-mL microcentrifuge tubes and 94.2 µL perchloric acid (70% vol/vol) was added to a final concentration of 0.6 M. Samples were incubated on ice for at least 30 min, and cell debris was removed by centrifugation at 4°C for 10 min at 13,500 × g. Supernatants were transferred to a 15-mL conical tube, and precipitated fractions were used for subsequent protein determinations. Then 1/5 volume (219 µL) of 2.5 M KHCO$_3$ was added to the supernatants to neutralize pH. The resulting salt precipitates were removed by centrifugation at 4°C for 10 min at 1510 × g using a 5810 R fixed-angle rotor (Eppendorf). Supernatants were transferred to new 2-mL microcentrifuge tubes and stored at −80°C until analysis by liquid chromatography-mass spectrometry (LC-MS). For protein quantitation, precipitated fractions were resuspended in 100 µL of 1 M NaOH and boiled at 100°C for 10 min. Samples were then cooled to room temperature prior to protein assay. Bradford protein assay was carried out on all samples in triplicate using a Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific). Bovine serum albumin (BSA) was used as a standard. Samples were analysed using a Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) (Hickman & Harwood, 2008; Thomann et al., 2006).

c-di-GMP separations were achieved through a 100 × 2.1 mm Syncronis C18 column (Thermo Fisher Scientific), eluted in a gradient system using isocratic elution protocol with 95% aqueous (2.5 mM ammonium acetate) and 5% organic (methanol). The flow rate was 0.2 mL/min and the cycle time was 10 min. c-di-GMP was detected by a Orbitrap Mass Analyzer on a Q Exactive Focus system (Thermo Fisher Scientific) in positive ionization mode. The ion spray voltage was 3.5 kV, sheath gas and aux gas pressures were at 45 psi and 10 arb, respectively. The mass-to-charge ratio (m/z) at 691.10214 molecular weight was used as the confirmatory signal and c-di-GMP quantification was done using selected-ion monitoring (SIM) mode. For a standard curve, 2.5, 5, 10, 20, 50, 100, and 500 nM pure c-di-GMP (Biolog) samples were analysed by the method described above. c-di-GMP levels were normalized to total protein per millilitre of culture. Data represent the average of three independent cultures and the error bars indicate the standard deviation.

4.10 | Pathogenicity assays

4.10.1 | Rice seed germination assay

The rice seed (Te Xianzhan, from the Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China) germination assay was based on the method described previously with minor modifications (Hussain et al., 2008). Twenty rice seeds were put into 10 mL of sterile water containing D. zeae EC1 or derivatives with 10$^2$ cfu and incubated for 5 h at room temperature. The rice seeds were rinsed three times with sterile water and transferred onto the top of two moistened filter papers placed on a sterilized plate at room temperature. Rice seeds were treated with the same volume of sterile water as a negative control. The rice seeds were then incubated at 28°C under 8 h dark/16 h light with supplementation of sterile water when necessary. The seed germination rate, and shoot and root length of rice plantlets were measured 7 days after incubation.

4.10.2 | Potato and radish pathogenicity assay

Potato (Solanum tuberosum 'Bintje') and radish (Raphanus sativus) were washed with tap water and dried on a paper towel. Potato tubers and radish roots were sliced evenly to about 5 mm thickness. The potato slices were washed three times with sterile water and dried on the sterilized filter paper for 30 min. The potato and radish slices were transferred onto two moistened filter papers on sterilized dishes and 1 µL of bacterial cells at OD$_{600} = 0.5$ in LB medium was added to the centre of the sliced potato tubers and radish roots. The potato and radish slices were incubated at 28°C for 24 h. Symptom development was observed and measured regularly, and each assay was repeated at least three times with triplicates.

4.11 | Statistic analysis

Each experiment was conducted with triplicates and repeated at least three times unless otherwise indicated. For easy comparison, certain data of mutants were normalized to those of the wild-type EC1, which were arbitrarily set as 100%. The paired two-tailed Student’s t test and significantly different values (analysis of variance, p < 0.05) were performed between the wild-type EC1 and its derivatives by using Prism v. 5.0 software (GraphPad).

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AUTHOR CONTRIBUTIONS

M.L. and L.Z. conceived the study. L.Z. and X.Z. supervised the study. M.L., M.H., Y.C., Q.Y., C.D., and S.Y. performed the experiments. J.L. and J.Z. analysed the data. M.L. drafted the manuscript. L.Z. and X.Z. revised the manuscript. All authors contributed to the revisions.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.
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**Supporting Information**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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