SlyA regulates phytotoxin production and virulence in *Dickeya zeae* EC1

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SUMMARY

Dickeya zeae is a causal agent of rice root rot disease. The pathogen is known to produce a range of virulence factors, including phytotoxic zeamines and extracellular enzymes, but the mechanisms of virulence regulation remain vague. In this study, we identified a SlyA/MarR family transcription factor SlyA in *D. zeae* strain EC1. Disruption of *slyA* significantly decreased zeamine production, enhanced swimming and swarming motility, reduced biofilm formation and significantly decreased pathogenicity on rice. Quantitative polymerase chain reaction (qPCR) analysis confirmed the role of SlyA in transcriptional modulation of a range of genes associated with bacterial virulence. *In trans* expression of *slyA* in *expl* mutants recovered the phenotypes of motility and biofilm formation, suggesting that SlyA is downstream of the acylhomoserine lactone-mediated quorum sensing pathway. Taken together, the findings from this study unveil a key transcriptional regulatory factor involved in the modulation of virulence factor production and overall pathogenicity of *D. zeae* EC1.

Keywords: *D. zeae*, regulation, rice foot rot, SlyA, zeamines.

INTRODUCTION

Dickeya zeae, previously named *Erwinia chrysanthemi* pv. *zeae*, is the causal agent of maize stalk rot and rice foot rot diseases in many countries and regions (Goto, 1970; Hussain *et al.*, 2008; Nassar *et al.*, 1994; Samson *et al.*, 2005; Sinha and Prasad, 1977). Outbreaks of the disease cause significant economic losses in rice and banana (Hussain *et al.*, 2008; Zhang *et al.*, 2013). The constant occurrence of *D. zeae*-associated diseases has received much attention and promoted research on the pathogenic mechanisms of this emerging pathogen. Concurrent with this trend, the genomes of several *D. zeae* strains have recently been sequenced

(Bertani *et al.*, 2013; Li *et al.*, 2012; Zhang *et al.*, 2013; Zhou *et al.*, 2015), which might substantially facilitate further investigations of the virulence and regulatory mechanisms of this important bacterial species.

Dickeya zeae is one of the six species belonging to the newly established genus, i.e. *Dickeya* gen. nov., which were reclassified from the *Erwinia chrysanthemi* complex (Samson *et al.*, 2005). Among the various *Dickeya* strains, the bacterial isolates that mainly infect dicotyledons, such as *D. dadantii* 3937 and EC16, have been studied extensively. These pathogens cause bacterial soft rot disease in a wide variety of economically important crops in various geographical regions. The hallmark of this disease is an area of 'mushy' tissues at the site of infection, which may eventually lead to the collapse of the entire plant (Barras *et al.*, 1994; Collmer and Bauer, 1994). As expected for soft rot symptoms, the pathogens produce a large array of plant cell wall-degrading enzymes, including different types of pectinases (Collmer and Bauer, 1994; Hugouvieux-Cotte-Pattat *et al.*, 1996; Robert-Baudouy *et al.*, 2000), proteases (Boyd and Keen, 1993; Ghigo and Wandersman, 1992; Shevchik *et al.*, 1998), feruloyl esterases (Hassan and Hugouvieux-Cotte-Pattat, 2011) and cellulases (Reverchon *et al.*, 1994), which collectively contribute to bacterial infections (Reverchon and Nasser, 2013; Zhou *et al.*, 2015). In addition, a catechol-type siderophore and a blue pigment indigoidine are also implicated in bacterial systemic invasion (Enard and Expert, 2000; Enard *et al.*, 1988; Expert and Toussaint, 1985; Reverchon *et al.*, 2002). The pectate lyases, which are the main virulence factors of *Dickeya* spp., are regulated by a complex network of regulatory systems. The key regulator appears to be the transcriptional repressor KdgR, whose repression is released by the presence of pectin degradation products, such as 2-keto-3-deoxygluconate (Robert-Baudouy *et al.*, 2000). In addition, other master regulators, including PecS, PecT, H-NS, Fis, Pir, the GacA/S two-component system, a newly identified quorum sensing (QS) system Vfm and a SlyA/MarR family regulator MfbR, are also known to be associated with the regulation of pectinase genes (Nasser *et al.*, 2013; Nomura *et al.*, 1998; Reverchon *et al.*, 2010; Yang *et al.*, 2008).

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The characterization of the pathogenic mechanisms of *D. zeae* was started a few years ago. Whilst working on an acylhomoserine lactone (AHL)-mediated QS system, we noted that *D. zeae* strain EC1, even at a very low population density, showed a strong inhibitory activity against rice seed germination, whereas *D. dadantii* strain 3937 did not display any inhibitory activity, even at a five-fold higher population density than strain EC1 (Hussain *et al.*, 2008). These findings suggest that a strain EC1-specific virulence factor(s) may account for this inhibitory activity. Our subsequent studies led to the identification of two phytotoxins, i.e. zeamine and zeamine II, with strong antimicrobial activities and a toxic effect against rice seed germination (Cheng *et al.*, 2013; Liao *et al.*, 2014; Wu *et al.*, 2010; Zhou *et al.*, 2011). Zeamine production is encoded by the *zms* gene cluster, and deletion of *zmsA*, which encodes a polyketide synthase, abolished zeamine production and the bacterial virulence on rice seeds (Cheng *et al.*, 2013; Zhou *et al.*, 2011, 2015). These findings suggest that the phytotoxic zeamines are the key virulence factors of *D. zeae* EC1, but it is not yet clear how zeamine production is regulated.

In this study, we report the identification and characterization of another SlyA/MarR family transcription factor, which was designated as SlyA based on its similarity to the transcriptional regulator SlyA from the closely related pathogen *D. dadantii* 3937 (Haque *et al.*, 2009). We showed that SlyA regulates diverse biological functions, including the production of zeamines and extracellular hydrolytic enzymes, cell motility, formation of cell aggregates and virulence on rice seeds. These findings suggest that SlyA is a key regulator that plays a global role in the regulation of *D. zeae* EC1 physiology and virulence.

RESULTS

Molecular characterization of a zeamine-deficient mutant EM53

We have shown previously that zeamines are potent antibiotics against *Escherichia coli* and other bacterial pathogens (Liao *et al.*, 2014; Wu *et al.*, 2010). For the identification of the genes that encode the regulation of zeamine production, about 30 000 transposon mutants of strain EC1 were screened for altered activities in the inhibition of *E. coli* growth. EM53, one of the mutants showing substantially reduced production of zeamines (Fig. 1A), was selected for further characterization in this study. Time course analysis showed that the mutant EM53 grew marginally more slowly than the wild-type strain EC1 at the exponential growth phase, but caught up at the late stationary phase (Fig. 1B), suggesting that the compromised production of zeamines in EM53 is unlikely to be caused by a growth defect. Analysis of the DNA sequence revealed that the transposon was inserted at nucleotide 126 of a gene designated as *slyA* (Fig. S1A, see Supporting Information) based on the high similarity of its product to the SlyA

Fig. 1 EC1 produces an antibiotic-like toxin zeamine, which requires a functional transcriptional regulator SlyA. (A) Plate bioassay of EC1, the Tn5 insertion mutant EM53, its complementary strain EM53/pslyA and the related *Dickeya dadantii* strain 3937. Forty microlitres of boiled bacterial cultures after 12 h (top row) and 24 h (bottom row) in LS5 medium were added to each well in an LB plate overlaid with *Escherichia coli* DH5α. The clear inhibition zone surrounding the well indicates antibiotic-like toxin activity. The photographs were taken 24 h after incubation at 37 °C. (B) The growth curves of EC1 and its derivatives in liquid LS5 medium at 28 °C. OD, optical density.

homologues in *D. dadantii* 3937 (YP_003883436.1, 98% identity) and *Serratia marcescens* (Q9RA96, 92% identity). *In trans* expression of the wild-type *slyA* restored zeamine production in the mutant EM53 (Fig. 1A), indicating the key role of the transcription factor SlyA in the regulation of zeamine biosynthesis.

SlyA is a global transcriptional regulator modulating about 25% of the bacterial genome

In order to fully understand the role of SlyA in *D. zeae*, we compared the gene expression of the *slyA*-deletion mutant, which showed a similar phenotype in zeamine production to the transposon mutant EM53, and the wild-type strain EC1. Of the predicted 4154 genes in the whole genome, about 25% were significantly [false discovery rate (FDR) ≤ 0.001 and fold change > 2] affected by *slyA* deletion, including 653 genes down-regulated and 395 genes up-regulated (Table S4, see Supporting Information). Table S3 (see Supporting Information) shows the selected genes

under the regulation of SlyA, most of which were reported to be associated with the virulence of *Dickeya* species. As expected, of the 18 genes in the *zms* gene cluster associated with the biosynthesis of zeamines (Cheng *et al.*, 2013; Zhou *et al.*, 2011, 2015), 14 were significantly reduced in transcript level by 2.3–23.3-fold, and the remaining four were reduced by over 1.4-fold (Table S3). The other affected genes include those encoding cell wall-degrading enzymes, exopolysaccharide (EPS) biosynthesis, five types of secretion system, QS signals, cyclic-di-GMP signalling systems, transporters, transcriptional regulators and proteins involved in cell motility, stress and toxin resistance (Table S3). Some important genes associated with virulence in various functional groups were selected for quantitative polymerase chain reaction (qPCR) analysis, and the results confirmed the data of transcriptomic analysis (Table S3).

SlyA controls the transcription of genes encoding plant cell wall-degrading enzymes

Recently, we have reported that a *prtGDEFBCX* cluster, encoding four proteases and three type I secretion system (T1SS) components, PrtDEF, is located on the negative strand of the EC1 chromosome (Zhou *et al.*, 2015). By transcriptomic analysis, we found that the inactivation of *slyA* increased the expression of *prtX* and *prtC* with 21.71- and 3.37-fold change, respectively, and decreased the expression of *prtB* and *prtF* with 5.73- and 2.65-fold change, respectively (Table S3), whilst the plate assays showed a generally increased production of proteases (Fig. 2A). The negative modulation of SlyA on expression of *prtX* and *prtC* was confirmed by qPCR analysis (Table S3).

In the genome of EC1, only two genes encoding polygalacturonases were found, which are *pehK* and *pehX* (Zhou *et al.*, 2015). Transcriptomic analysis showed slightly increased expression of *pehK*, whereas qPCR showed higher expression (fold change, 2.150 ± 0.200), consistent with the semi-quantitative approximation of polygalacturonase activity (Fig. 2B).

Among the 14 genes encoding pectin degradation enzymes (Zhou *et al.*, 2015), five pectate lyase-encoding genes (*pnl*, *pell*, *pell*, *pelA* and *pelE*) and a pectate disaccharide-lyase-encoding gene (*pelX*) showed reduced expression with a fold change ranging from 2.04 to 5.02 (Table S3), highly consistent with the result of pectinase assay (Fig. 2C). Notably, T2SS, encoded by *out* genes, allowing the secretion of most pectinases (Lindeberg *et al.*, 1998), was also down-regulated (Table S3).

Complementation analysis showed that *in trans* expression of *slyA* in the deleted mutant Δ slyA partially rescued protease production and completely restored the production of polygalacturonases and pectinases (Fig. 2), consolidating the divergent roles of SlyA in the regulation of these extracellular enzymes.

Fig. 2 Mutation of *slyA* affects the production of extracellular enzymes in *Dickeya zeae* EC1. Enzyme activity assays were performed for proteases (A), polygalacturonases (B) and pectate lyases (C) using solid medium supplemented with the corresponding substrates. Plates were prepared by pouring about 30 mL of medium into 12 × 12-cm² plates. Bacterial cells were grown to an optical density at 600 nm (OD₆₀₀) of 1.2, and samples of 40 μ L of each bacterial culture were added to each well. Plates were incubated overnight at 28 °C. After 10 h, pectate lyase and polygalacturonase assay plates were developed with 4 M HCl. Haloes around the wells caused by protease activity became visible in protease assay plates within 24 h without any further treatment. The diameters of the haloes were measured and compared with those of EC1 to semi-quantify the enzyme activity. WT, wild-type.

Mutation of *slyA* affects the cell motility of *D. zeae* EC1

Mutation of *slyA* in the mutants EM53 and Δ slyA drastically enhanced the bacterial swimming motility by about two-fold (Fig. 3A), and increased the swarming motility by about four-fold (Fig. 3B), compared with the wild-type strain EC1. As SlyA has been determined recently to regulate the two flagellar rotation genes *motA* and *motB* by controlling PhoP under low-magnesium

Fig. 3 Mutation of *slyA* results in significantly enhanced bacterial swimming motility and swarming motility through an acylhomoserine lactone (AHL)-mediated quorum sensing (QS) system. The QS mutant WM3, deficient in *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL) production (Hussain *et al.*, 2008), was used as a control. A supplement of 2 μ M OHHL (filled bars) restored the swimming (A) and swarming (B) phenotypes of WM3, but not of the *slyA* mutants EM53 and Δ slyA, whereas *in trans* expression of *slyA* fully restored the swimming (C) and swarming (D) motilities of the QS mutants WM3 (Tn5 insertion mutant) and Δ expl (deletion mutant). The experiments were repeated four times and the data are the averages ($\pm$ standard deviation) of three repeats.

conditions in *D. dadantii* 3937 (Haque *et al.*, 2015), we tested the transcriptional levels of *phoP*, *motA* and *motB* in strain EC1. The results showed that all of these genes have similar transcriptional levels in the *slyA* mutant and wild-type EC1 (Table S3), implying that SlyA in *D. zeae* EC1 regulates cell motility in a different pathway from that in *D. dadantii* 3937.

As the motility phenotypic changes are reminiscent of those caused by mutation of *expl*, which encodes an AHL synthase

(Hussain *et al.*, 2008), we included the *expl* mutant WM3 as a control. The results showed that disruption of *slyA* generated a similar increase in motility as the QS mutant WM3 under the same experimental conditions (Fig. 3A,B). Given that the cell motility of strain EC1 is under the negative control of AHL-type QS signals (Hussain *et al.*, 2008), we tested whether exogenous addition of *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL), which appears to be the sole AHL signal produced by EC1 (Hussain *et al.*, 2008), could suppress the cell motility of the *slyA* mutants EM53 and Δ slyA. The results showed that the addition of OHHL suppressed both the swarming and swimming motility of the QS mutant WM3. In contrast, the QS signal had no effect on the cell motility of the mutants EM53 and Δ slyA, whereas *in trans* expression of *slyA* in the deletion mutant Δ slyA fully restored the cell motility of the mutants to the wild-type level (Fig. 3A,B). These data suggest that AHL-mediated QS cannot be downstream of SlyA. We expressed *slyA* in the QS mutants WM3 and Δ expl (the deletion mutant). Interestingly, the results showed that *in trans* expression of *slyA* could fully restore the cell motility of the mutants (Fig. 3C,D), suggesting that SlyA may function downstream of the AHL-mediated QS regulatory pathway associated with cell motility.

SlyA is essential for *D. zeae* biofilm formation

A previous study has shown that EC1 forms biofilms (cell aggregates) in liquid culture, and deletion of *expl* encoding AHL production abolishes biofilm formation in LB medium and substantially attenuates biofilm formation in SOBG medium (Hussain *et al.*, 2008). Yap *et al.* (2005) explored the potential for multicellular behaviour and found that *Dickeya* strains could form biofilms and pellicles in rich medium. Therefore, we used SOBG medium with richer ingredients (see Experimental Procedures) to further test biofilm formation. Similar to the *expl* mutant WM3, mutation of *slyA* also abrogated the bacterial ability to form cell aggregates and biofilms in SOBG medium, which was restored to the wild-type level by *in trans* expression of *slyA* in the mutant (Fig. 4A,B). In contrast, although the addition of the AHL signal OHHL to the *expl* mutant WM3 fully restored its ability to form cell aggregates and biofilms, the same addition of the QS signal could not rescue the formation of cell aggregates and biofilms in the *slyA* mutants (Fig. 4A,B). Interestingly, *in trans* expression of *slyA* in the QS mutants WM3 and Δ expl partially restored the biofilm formation of the mutants (Fig. 4C).

Mutation of *slyA* significantly decreases the pathogenicity of *D. zeae* on rice seeds

The pathogenicities of *D. zeae* strain EC1 and its derivatives were determined using the previously established rice seed germination assay (Hussain *et al.*, 2008). Rice seeds were mixed with fresh bacterial culture for 5 h prior to washing with sterilized water, and the seed germination rate was determined 1 week after

Fig. 4 SlyA regulates the cell aggregation (A) and biofilm formation (B, C) of *Dickeya zeae* downstream of acylhomoserine lactone (AHL)-mediated quorum sensing (QS) regulation (C). When necessary, *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL) was added to a final concentration of 10 μM (A, B). After 6 h of shaking in SOB medium at 28 °C, samples were collected for observation and photographs were taken at ×200 under an Olympus BX50 microscope (A). Overnight bacterial cultures were inoculated into SOB broth to an optical density at 600 nm (OD₆₀₀) of 0.01, 100 μL of which were added to each well at 28 °C with shaking at 150 rpm for 18 h. Attached bacterial cells were dyed with 200 μL of 1% crystal violet (w/v), rinsed with water, dried, dissolved in 200 μL of 95% ethanol and quantified by measurement of the absorbance at 570 nm (B, C). The experiments were repeated three times and the figure shows a representative set of photographs.

incubation in a growth chamber. Challenge of rice seeds with the wild-type strain EC1 or complemented strains inhibited both shoot and root germination (Fig. 5B,E,F), whereas rice seeds treated with the *slyA* mutants germinated and grew normally, similar to the LB control (Fig. 5A,C,D). Quantitative analysis of 60 rice seeds

showed that mutation of *slyA* resulted in a decrease of more than 96% in bacterial virulence, as indicated by the increased rate of rice seed germination (Fig. 5G).

DISCUSSION

Dickeya zeae strain EC1, different from other *Dickeya* spp., is able to infect both dicotyledons and monocotyledons. Our previous studies have shown that the pathogen produces phytotoxins, i.e. zeamine and zeamine II, as key virulence determinants in the infection of host plants (Cheng *et al.*, 2013; Zhou *et al.*, 2011). In this study, we found that the production of zeamines is positively regulated by a MarR family transcription factor SlyA, as null mutation of *slyA* substantially reduced the production of the phytotoxins (Fig. 1A). Transcriptomic analysis indicated that the regulation of *zms* genes occurs at the transcriptional level (Table S3). In addition, SlyA is also involved in the regulation of other biological functions associated with bacterial pathogenesis, including the production of proteases, polygalacturonases and pectate lyases (Fig. 2), bacterial motility (Fig. 3) and biofilm formation (Fig. 4). In particular, disruption of the gene *slyA* in *D. zeae* EC1 abolished its inhibitory activity against rice seed germination (Fig. 5). Corresponding to the changed phenotypes, genes encoding associated virulence factors were found to be regulated by SlyA from the results of transcriptomic analysis.

Generally, SlyA positively regulates about 653 genes and negatively controls the expression of over 395 genes encoding various biological functions, including the biosynthesis of zeamines, production of cell wall-degrading enzymes, efflux systems, c-di-GMP metabolism and cell motility. It activates most secretion systems, such as T2SS (two- to three-fold), T3SS (two- to 36-fold), T4SS (two- to 50-fold) and T6SS (two- to 32-fold) (Tables S3 and S4). These findings thus depict a global regulator that has both a positive and negative effect on *D. zeae* virulence gene expression. This dual role has also been found in another MarR family transcriptional regulator, PecS, which acts as a repressor of *pel* genes and an activator of *peh* genes (Nasser *et al.*, 1999), illustrating the complexity of the regulation of MarR family regulators.

More interestingly, SlyA also affects the expression of many housekeeping genes, including several operons encoding ribosomal proteins, such as AJC64774.1 to AJC64779.1, involved in the formation of 50S ribosomal proteins, AJC67870.1 to AJC67903.1, related to numerous 30S and 50S ribosomal proteins, and AJC66383.1 to AJC66385.1, responsible for the synthesis of exodeoxyribonuclease V subunits (Table S3). To our knowledge, this is the first report indicating that SlyA widely modulates the expression of ribosomal protein-encoding genes.

SlyA also affects the expression of several transcriptional regulators, such as another MarR family regulator MfaR, a Fis family

Fig. 5 Disruption of *slyA* in strain EC1 abolishes its inhibitory activity on rice seed germination. Rice seeds were treated for 5 h with LB medium (A) or bacterial culture [optical density at 600 nm (OD_{600}) of 1.2] of the wild-type strain EC1 (B), the mutants EM53 (C) and Δ slyA (D) and the complemented strains EM53/pslyA (E) and Δ slyA/pslyA (F), and then washed and incubated at 28 °C under 16 h light and 8 h dark conditions. (E) Quantitative analysis of rice seed germination. The experiment was repeated three times with 20 seeds each time. Photographs were taken 1 week after inoculation. The data are averages of all 60 randomly selected seeds, and error bars indicate standard deviation.

transcriptional regulator, several XRE family transcriptional regulators etc., which, themselves, control sets of genes. Thus, some genes affected by the *slyA* mutation could be directly attributed to SlyA, but several others are probably indirectly affected, which should be confirmed by further study.

The SlyA homologues are widely distributed in prokaryotes. Genome sequence analysis reveals more than 300 putative members of the MarR/SlyA family in 58 species of bacteria and Archaea (Ellison and Miller, 2006). Therefore, one bacterial genome may encode several MarR/SlyA-type transcription factors of various similarities. Among the 17 reported MarR family regulators in *D. dadantii* 3937 (Reverchon *et al.*, 2010), at least nine MarR family members were found in *D. zeae* strain EC1, including EmrR, PecS, SlyA, OhrR, MfaR, MfbR, MfgR, MfiR and MflR (Table S5,

see Supporting Information). Members of the MarR/SlyA family of transcription factors are implicated in the regulation of various biological functions. In the well-characterized *D. dadantii* 3937, three SlyA/MarR-type transcription factors have been characterized. Among them, PecS controls pectinase, cellulase and blue pigment production (Reverchon *et al.*, 1994), SlyA positively regulates the production of pectate lyase, biofilm formation and T3SS (Haque *et al.*, 2009; Zou *et al.*, 2012), and MfbR activates the genes encoding cell wall-degrading enzymes, such as proteases and pectate lyases (Reverchon *et al.*, 2010). Although it is not yet known how SlyA affects proteases and polygalacturonases in *D. dadantii* 3937, the general regulatory mechanism of SlyA appears to be conserved in *D. dadantii* 3937 and *D. zeae* EC1, as it plays a similar positive regulatory role in the modulation of

pectate lyase production and biofilm formation. Although *D. dadantii* lacks the genes encoding the biosynthesis of zeamines (Zhou *et al.*, 2015), it has been reported that a SlyA family regulator Rap is involved in the regulation of the biosynthesis of the antibiotics prodigiosin and carbapenem in *Serratia* sp. (Slater *et al.*, 2003). Recently, it has been shown that, in addition to *D. zeae*, *Serratia plymuthica* also produces the antibiotic zeamines (Masschelein *et al.*, 2013). It would be interesting to test whether SlyA plays a similar role in the regulation of zeamine biosynthesis in these two distantly related bacterial species.

In the MarR family, SlyA orthologues have been characterized: e.g. SlyA in *Salmonella* species, which shares 73.72% homology to that in EC1, is required for resistance to oxidative stress and survival in macrophages (Buchmeier *et al.*, 1997; Libby *et al.*, 1994); RovA in *Yersinia pseudotuberculosis* and Hor in *Erwinia carotovora*, which share 71.78% and 85% similarity, respectively, to SlyA in EC1 are involved in the production of virulence factors (Basset *et al.*, 2003; Heroen *et al.*, 2004); and SlyA with 71.04% identity controls the production of haemolysin cytolytic toxin in *E. coli* K-12 (Lithgow *et al.*, 2007).

The molecular mechanisms by which SlyA controls gene expression have been well characterized in *Salmonella enterica* (Okada *et al.*, 2007). Site-directed mutagenesis reveals several key amino acid residues of SlyA that are critical for its transcriptional regulatory activity. Among them, seven residues (L63, V64, R65, L67, L70, R86 and K88) are associated with DNA binding and two residues (L12 and L126) are required for efficient dimer formation (Okada *et al.*, 2007). These nine key residues are perfectly conserved in SlyA of *D. zeae* EC1 (Fig. S1), suggesting that the regulator might also form a dimer and interact with the consensus SlyA binding motif (T-T-A-G/T-C/T-A-A-G/A-C/A-T-A-A) at the promoter regions of its regulated genes (Okada *et al.*, 2007; Stapleton *et al.*, 2002). These findings, together with the results of transcriptomic analysis, may facilitate further dissection of the SlyA regulon and characterization of its regulatory pathways. Interestingly, we found a consensus 'TTATTAACAATAA' motif located 41 to 29 bp upstream of the start codon of *zmsA*, indicating that SlyA directly controls *zmsA* transcription by interacting with its promoter. In *D. dadantii* 3937, SlyA has been demonstrated to bind to the promoter region of the *phoP* gene containing the putative SlyA binding motif 'ATAGTTCATAA' (Haque *et al.*, 2009), whereas a DNA consensus 'ATAATTCATAA' is located at 55 to 44 bp upstream of the ATG start codon of *phoP* in EC1. However, the transcriptional level showed a non-significant (-1.36 -fold and 0.895 ± 0.025 gene expression of EC1) change, suggesting that SlyA might modulate the virulence of EC1 via a different pathway from that in *D. dadantii* 3937.

As SlyA plays similar roles in the regulation of bacterial cell motility and biofilm formation to the AHL QS signal (Hussain *et al.*, 2008), we deduced that they are possibly located in the

same pathway of virulence regulation. We therefore tested whether the QS system could act downstream of SlyA. However, the addition of AHL signals to the *slyA* mutant failed to rescue the mutant phenotypes in cell motility and biofilm formation (Figs 3 and 4A,B). In addition, disruption of *slyA* in *D. zeae* EC1 did not change the rate of AHL production (Fig. S2, see Supporting Information). Furthermore, mutation of *slyA* abolished the formation of cell aggregates, whereas the QS mutants were still able to generate cell aggregates, although smaller than those of the wild-type (Fig. 4A). Cumulatively, the above findings suggest that the QS system is not downstream of SlyA. On the contrary, we transferred the *slyA*-harbouring complementary plasmid into the QS mutants WM3 and Δexl (the deletion mutant), and found that *in trans* expression of *slyA* fully restored the cell motility (Fig. 3C,D) and partially recovered the biofilm formation (Fig. 4C) of the QS mutants; furthermore, the expression of *slyA* showed a 3.99-fold decrease in the QS mutant Δexl (from the RNA-seq result of Δexl), indicating that the AHL-mediated QS system directly controls *slyA* at the transcriptional level and is located upstream.

The identification of SlyA as a key regulator governing the biosynthesis of the phytotoxic zeamines and the ribosomal proteins in *D. zeae* EC1 presents a new addition to the expanding list of physiological functions regulated by SlyA/MarR-type proteins, which are a family of transcriptional regulators widely conserved in various human, animal and plant pathogens (Thomson *et al.*, 1997). Our previous studies have demonstrated that zeamines are the dominant virulence factors of *D. zeae* (Cheng *et al.*, 2013; Zhou *et al.*, 2011), but the mechanisms of regulation remain unclear. The identification of SlyA as a key regulatory factor controlling the production of zeamines and other virulence factors provides a useful stepping stone for further characterization of the signalling mechanisms and regulatory pathways with which *D. zeae* modulates the production of virulence factors during the invasion of host plants.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture media

The bacterial strains and plasmids used in this study are described in Table S1 (see Supporting Information). The *D. zeae* wild-type strain EC1, the QS mutants WM3 and Δexl , and *E. coli* strains have been described previously (Hussain *et al.*, 2008). *Escherichia coli* strains were routinely grown at 37 °C in LB medium (per litre contains 10 g bactotryptone, 5 g yeast extract and 10 g NaCl, pH 7.0). *Dickeya zeae* strains were grown at 28 °C in LB to test cell degradation enzyme activities, in SOB (per litre contains 20 g tryptone, 5 g yeast extract, 2.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g NaCl, 0.186 g KCl and 20 mL glycerol) (Yap *et al.*, 2005) for biofilm formation, on minimal medium (MM) agar plates (Hussain *et al.*, 2008) for conjugation and in LS5 medium (per litre contains 5.25 g K_2HPO_4 , 2.25 g KH_2PO_4 , 10.0 g sucrose, 3.6 g NH_4NO_3 , 1.0 g KCl and 0.25 g MgSO_4 , pH 7.0) (Liao *et al.*, 2014) for zeamine production. Antibiotics were added at the

following concentrations when required: gentamycin (Gm), 50 µg/mL; ampicillin (Ap), 100 µg/mL; kanamycin (Km), 50 µg/mL; tetracycline (Tc), 15 µg/mL.

Transposon mutagenesis

A mini Tn5 transposon, carried by the plasmid pTGN in *E. coli* S17-1, was introduced into the genome of strain EC1 by conjugal mating as described previously (Tang *et al.*, 1999). Conjugal mating was performed by mixing the overnight cultures of the corresponding bacterial strains on an LB agar plate incubated at 28 °C for 6 h. Transconjugants were then selected on MM plates containing 50 µg/mL Km and 50 µg/mL Gm.

DNA cloning and sequencing

The genomic DNA samples of the mutants were extracted with a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, USA). The DNA fragments flanking the Tn5 insert were cloned by thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) as described previously (Zhou *et al.*, 2011) using the nested primers Gt-447, Gt-464 and Gt-487 and an arbitrary primer AD6, listed in Table S2 (see Supporting Information), designed on the basis of the Tn5 sequence (Tang *et al.*, 1999). For sequencing the TAIL-PCR products, a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used and the reaction was carried out on an MJ Research PTC 100 Thermal Cycler (Foster City, CA). Sequence determination was carried out by the DNA Sequencing Facility using an automated DNA sequencer 3700 (ABI, CA), according to the manufacturer's protocol. Sequences were analysed using National Center for Biotechnology (NCBI) BLAST tools (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequence obtained in this study was deposited in GenBank with the following accession number: EU443200.

In-frame deletion and complementation of *slyA*

To knock out the open reading frame (ORF) of *slyA*, approximately 600-bp fragments located upstream and downstream of *slyA* from EC1 genomic DNA were amplified using the primer pairs *slyA*-1/*slyA*-2 and *slyA*-3/*slyA*-4 (Table S2), respectively, and the two fragments were fused using the primer pair *slyA*-1 and *slyA*-4. The fusion fragment and suicide plasmid pKNG101 were then digested with *Bam*HI and *Spe*I, respectively, ligated together and transformed into *E. coli* CC118. For the generation of the in-frame deletion mutant, triparental mating was performed using the methods described previously (Cheng *et al.*, 2013). Mutants were selected on MM plates supplemented with 5% sucrose and then confirmed by DNA sequencing.

For the generation of the complementary strain, the fragment containing 166 bp of promoter sequence and the ORF of *slyA* was amplified using the primers *slyA*-BamHI and *slyA*-HindIII (Table S2). The PCR products were purified and subjected to digestion with *Bam*HI and *Hind*III. The products were purified and ligated into the pUC19 vector digested with *Bam*HI and *Hind*III. The ligation products were then transformed into *E. coli* DH5α competent cells, and the transformants were selected on an LB plate supplemented with 100 µg/mL Ap. The expression construct was confirmed by DNA sequencing before introduction into the mutants EM53, Δ*slyA*, WM3 or Δ*exl* by triparental mating. The complemented strains

were selected on MM plates supplemented with 100 µg/mL Ap, and confirmed by PCR analysis.

RNA preparation

Overnight cultures of *D. zeae* strains were added to fresh LB medium or LS5 medium (for expression analysis of *zmsA* and *zmsK*) at a ratio of 1 : 100 and grown to an optical density at 600 nm (OD₆₀₀) of 1.0. Bacterial cells were harvested by centrifugation and RNA molecules were isolated using an RNeasy Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. The quantity of RNA was measured using a NanoDrop, Wilmington, DE ND-100 spectrophotometer at 260 nm after removal of contaminating genomic DNA with DNase I (Roche, Germany) at 28 °C for 1.5 h. The absence of DNA contamination was confirmed by PCR using the primer pair 16S-F/16S-R (Table S2) and visualized on an agarose gel.

RNA-seq and bioinformatics analysis

Ribosomal RNA (rRNA) was removed using a Ribo-Zero Magnetic Gold Kit (Epicentre, USA), and mRNA was enriched and fragmented into 200 nucleotides with fragmentation buffer. The first strands of cDNA were synthesized using random hexamers, and the second strands were then synthesized by adding buffer, deoxynucleoside triphosphates (dNTPs), RNase H and DNA polymerase I. After purification with a QIAquick PCR Kit (QIAGEN, Germany), the ends of the double-strand cDNAs were repaired, adding adenine and sequence adaptors. Fragments were purified and collected after agarose gel electrophoresis, and subsequently amplified using PCR. The products were then diluted at a concentration of 1 ng/µL. The insert size of the cDNAs was analysed using an Agilent 2100 (Agilent Genomics, CA). Finally, a library of cDNAs of 200 bp in size was constructed and bound on the surface of the flow cell using TruSeq PE Cluster Kit v3-cBot-HS reagents (Illumina, CA) for amplification to create clonal clusters, and then sequenced on an Illumina HiSeq2500 platform (CA) using PE100 strategy.

Raw data were obtained by base calling using CASAVA software, and sequences of low quality ($Q \leq 5$), with stained adaptors ($bp \geq 5$) or matched to rRNAs were removed to obtain clean reads, which were then aligned with the reference genome of *D. zeae* EC1 (CP006929.1) using Tophat software v2.0.12, (Center for Computational Biology, USA). Gene expression differentiation was calculated using the number of reads per kilobase per million mapped reads (RPKM), and analysed using DESeq. Genes with $|\log_2 \text{Ratio}| \geq 1$ and $q \leq 0.05$ were selected as differentially expressed genes.

Zeamine bioassay

The bioassay plates were prepared by pouring about 20 mL of LB agar medium into 12 × 12-cm² plates, which, after solidification, were overlaid with 10 mL of 1% agarose (42 °C) containing about 2×10^8 cells of fresh *E. coli* DH5α. The wells of 4 mm in diameter were punched in the bioassay LB agar plates. The supernatants of overnight bacterial cultures in LS5 medium (Liao *et al.*, 2014) were collected after centrifugation and boiled for 10 min. For each sample, duplicates of 40 µL supernatants were taken and added to bioassay wells. Plates were incubated overnight at 37 °C. A clear inhibitory zone on the background of the *E. coli* DH5α growth lawn

was indicative of the antimicrobial activity of zeamines (Wu *et al.*, 2010; Zhou *et al.*, 2011).

AHL bioassay

Agrobacterium tumefaciens CF11 containing a *tra-lacZ* fusion gene was used as an AHL biosensor. Briefly, 20 mL of MM supplemented with X-Gal (40 µg/mL) was poured onto a plate. After solidification, the medium was cut into separate slices (1 cm in width). Fresh cultures (1 µL) of the AHL biosensor at OD₆₀₀ = 0.1 were spotted progressively onto the slices, and cultures (1 µL) of *D. zeae* EC1 and its derivatives at OD₆₀₀ = 1.0 were spotted onto the top of the slices. The plates were incubated at 28 °C for 24 h. The blue spots of the biosensor indicated AHL activity (Hussain *et al.*, 2008). Synthetic OHHL (2 µg/mL) was used as a control.

Extracellular degrading enzyme activity

The activities of cell wall-degrading enzymes were determined according to previously described methods (Barras *et al.*, 1987; Chatterjee *et al.*, 1995; Scott-Craig *et al.*, 1990). The compositions of the media for the assay of enzymatic activity were as follows: pectate lyase assay medium (per litre): 10 g polygalacturonic acid (PGA), 10 g yeast extract, 8 g agarose, 0.38 µmol CaCl₂ and 100 mmol Tris-HCl, pH 8.5, pH 7.0; polygalacturonase assay medium (per litre): 5 g PGA, 2 g sucrose, 2 g (NH₄)₂SO₄ and 15 g agar, pH 7.0; protease assay medium (per litre): 10 g skimmed milk, 5 g bactotryptone, 2.5 g yeast extract and 5 g NaCl. The bioassay plates were prepared by pouring about 30 mL of medium onto 12 × 12-cm² plates, and wells of 4 mm in diameter were punched after solidification. Bacterial cells were grown to OD₆₀₀ = 1.2 and samples of 40 µL of bacterial cultures were applied to the wells. Plates were incubated overnight at 28 °C. After 10 h, pectate lyase and polygalacturonase assay plates were developed with 4 M HCl, and protease activity was indicated by haloes around the wells within 24 h without any further treatment. The experiments were repeated at least three times with duplicates.

Swimming, swarming, cell biofilm and aggregation assay

For the determination of swimming motility, 1 µL of an overnight bacterial culture was spotted onto the centre of a plate containing about 20 mL of semisolid bactotryptone agar medium (per litre contains 10 g bactotryptone, 5 g NaCl and 3 g agar). The plates were incubated at 28 °C for 6 h before measurement of the diameters of the bacterial swimming zone. The swarming motility was assayed under the same conditions, except for the medium (per litre contains 5 g peptone, 3 g yeast extract and 4 g agar). Each experiment was repeated at least three times in triplicate.

For the observation of cell aggregation, overnight starter cultures of strain EC1 and its derivatives were diluted to OD₆₀₀ = 0.5, and 50 µL of each bacterial dilution were added to a 50-mL Falcon tube containing 10 mL of fresh LB or SOBG medium. The tubes were incubated at 28 °C with shaking at 250 rpm for 6 h before microscopic examination. The experiments were repeated three times and representative figures are presented.

For the observation of biofilm formation, 2 µL of overnight bacterial culture were added to 100 µL of SOBG broth in the well of a 96-well plate, and grown at 28 °C with shaking at 150 rpm for 18 h. The culture

was removed and 200 µL of crystal violet (w/v) were added. After staining for 5 min, the well was rinsed three times with water and dried at room temperature. Finally, 200 µL of 95% ethanol were added to dissolve the attached bacterial cells. The quantity of biofilm formation was determined by measurement of the absorbance at 570 nm.

Rice seed germination assay

The assay was based on the method described previously with minor modifications (Hussain *et al.*, 2008). Briefly, 20 seeds of rice variety IR24 were added to 5 mL of a bacterial culture (OD₆₀₀ = 1.2) and incubated at room temperature for 5 h. The rice seeds were then washed three times with sterilized water and transferred onto two moistened Whatman no. 3 filter papers in a Petri dish. The seeds were then incubated at 28 °C under 16 h light and 8 h dark, and sterilized water was added when necessary. Rice seeds were incubated with the same amount of LB medium as a blank control. The rate of seed germination was determined 1 week after treatment. The experiment was repeated three times.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

To analyse the expression differentials of genes corresponding to the phenotypic changes, qRT-PCR analysis was performed using a iQ SYBR Green Supermix (Bio-Rad, USA) on the CFX 96 Real-time PCR equipment (Bio-Rad, USA). The primers listed in Table S1 were designed using Primer Quest software (<http://test.idtdna.com/Scitools/Applications/Primerquest/>) based on the gene sequences of *D. zeae* ZJU1202 (Li *et al.*, 2012) obtained from NCBI. The amplification conditions were as follows: 95 °C for 15 s in the initial activation step, denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s with 50 cycles. All treatments were performed in triplicate. The ratio of the specific gene expression level in the *slyA* mutant compared with the wild-type EC1 was normalized to the level of expression of the 16S rRNA gene.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 The genome organization of *slyA* and similarity to its homologues. (A) The genome organization of the *slyA* locus. The relative transposon insertion site and transcriptional directions are indicated. (B) A conserved MarR-type HTH domain was predicted to be present in SlyA analysed with SMART (<http://smart.embl-heidelberg.de>). (C) Peptide sequence alignment of SlyA with homologues. SlyA-3937, SlyA of *Dickeya*

dadantii 3937 (NCBI accession no. YP_003883436.1); SlyA-Sma, SlyA of *Serratia marcescens* (NCBI accession no. Q9RA96); SlyA-Ecc, SlyA of *Erwinia carotovora* ssp. *carotovora* (NCBI accession no. Q9RB09); SlyA-See, SlyA of *Salmonella enterica* (NCBI accession no. P61090). The predicted HTH DNA-binding motif is marked by the line above the sequence, and the residues implicated in dimerization (L12, L126) and in DNA binding (L63, V64, R65, L67, L70, R86, K88) are highlighted. Asterisks (*), colons (:) and dots (.) indicate identical, highly similar and similar residues, respectively.

Fig. S2 Inactivation of *slyA* gene of *Dickeya zeae* EC1 has no impact on acylhomoserine lactone (AHL) quorum sensing signal production. Cultures of strain EC1 and its derivatives were spotted on the top of the minimal medium bar. The AHL biosensor strain CF11 was spotted on the rest of the bar, which turned blue in the presence of AHL signals. LB medium and synthetic *N*-(3-oxo-hexanoyl)-homoserine lactone (OHHL) were spotted as controls.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Primers used in this study.

Table S3 Selected genes regulated by SlyA.

Table S4 List of regulated genes of SlyA.

Table S5 Homologue of MarR family regulators in *Dickeya zeae* EC1 and *D. dadantii* 3937.